EFFECTS OF DIFFERENT ELECTRODES IN THE GROWTH OF SALT AND ETHANOL TOLERANT YEAST ISOLATED FROM MURCHA OF NEPAL

M.Sc. Thesis
2023 A.D.

Submitted to

CENTRAL DEPARTMENT OF BIOTECHNOLOGY

Tribhuvan University

Kirtipur, Kathmandu, Nepal

Dikshya Regmi

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Recommendation

This is to certify that the research work entitled **"EFFECTS OF DIFFERENT ELECTRODES IN THE GROWTH OF SALT AND ETHANOL TOLERANT YEAST ISOLATED FROM MARCHA OF NEPAL"** has been carried out by Mr/Ms Dikshya Regmi under my supervision.

This thesis work was performed for the partial fulfillment of the Master of Science in Biotechnology under the course code BT 621. The result presented here is his/her original findings. I/We, hereby, recommend this thesis for final evaluation.

……

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Certificate of Evaluation

This is to certify that this thesis entitled **"STUDY OF EFFECTS OF DIFFERENT ELECTRODES IN THE GROWTH OF SALT AND ETHANOL TOLERANT YEAST ISOLATED FROM MARCHA OF NEPAL"** presented to evaluation committee by Mr/Ms. Dikshya Regmi is found satisfactory for the partial fulfillment of Master of Science in Biotechnology.

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LIST OF ABBREVIATIONS

Bp: basepairs

DNA: Deoxyribonucleic acid

DNS: Dinitrosalicylic acid

D/W: Distilled water

dNTP: Deoxynucleoside triphosphate

HPLC: High Performance Liquid Chromatography

kDa: kilodalton

Ml: Mililiter

NAD: Nicotinamide Adenine Dinucleotide

PCR: Polymerase Chain Reaction

PYN: Peptone Yeast extract Nutrient

RNA: Ribonucleic acid

rRNA: ribosomal RNA

SDS: Sodium dodecyl sulfate

SDS-PAGE:Sodium dodecyl sulfate- Polyacrylamide gel electrophoresis

TBP: Tri-n-butyl phosphate

TEMED: Tetramethylethylenediamine

YEPD: Yeast extract Peptone Dextrose

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ABSTRACT

Many yeast species have the capability to transform various types of hexose sugars into ethanol through glycolysis. To determine the effects of different electrodes and applied voltage on glucose metabolism and ethanol production, various electrochemical combinations were tested using a 4-volt electric current. The results revealed that yeast exhibited the highest activity for ethanol production in the anode side of the graphite-graphite electrochemical cell and in the cathode side of the graphite-platinum electrochemical cell. Ferricyanide treatment did not show any significant increase in ethanol production. Nevertheless, when the yeast culture was kept at the ideal temperature of 28°C while undergoing ferricyanide treatment, a notable enhancement in yeast metabolism was witnessed in the graphite anode at 96 hrs. At this time point, the ethanol concentration increased from 16.74 ± 0.13 mg/mL at 72 hrs to 41.48 ± 0.10 mg/mL. However, the ethanol concentration dropped to 21.82 ± 0.12 mg/mL at 120 hrs. In the bioreactor, the highest production of ethanol by yeast was observed at 72 hrs, with an ethanol concentration of 58.76 ± 0.10 mg/mL. At 96 and 120 hrs, the ethanol concentration decreased to 32.29 ± 0.58 mg/mL and 22.66 ± 0.57 mg/mL, respectively. The results indicate that the application of a low level of voltage increased ethanol production in yeast. The use of electrochemical cells also resulted in better glucose metabolism and ethanol production in yeast compared to other general microbial techniques. The maximum percentage of ethanol produced 5.88. The presence of glucose and ethanol in the samples was confirmed via HPLC. Additionally, the 18s rRNA PCR product size was around 600 bp, and a protein of sizes 26kDa, 34kDa, and 43 kDa was observed in the sample. Overall, the study provides valuable insights into the effects of different electrochemical cells and setups on glucose and ethanol metabolism in yeast. Such research is crucial in providing necessary data and insights into the development of microbial fuel cells.

Keywords: Electrochemical cells, ethanol, graphite electrode, murcha, PCR, platinum electrode, SDS-PAGE

Chapter 1. Introduction

1.1. Introduction

Murcha aka marcha (Takeuchi et al., 2006; Sha et al., 2017) is a mixed dough inoculum used as a starter culture in fermentation for production of indigenous alcoholic beverages such as jaand or chyaang in various part of India, Nepal, and Bhutan (Tamang et al., 1996). Murcha is characterized as a dry, spherical or flattened substance, varying in color from creamy white to dusty white. It takes the form of a solid ball, with a diameter ranging from 1.9 to 11.8 cm and a weight ranging from 2.3 to 21.2 g. The microflora of Murcha consists of bacteria like *Pediococcus pentosaceus*, yeasts like *Saccharomycopsis fibuligera* and, Mucor species such as *Mucor circinelloides* and *Rhizopus chinensis*, and *Pichia anomala* (Tamang and Sarkar, 1996). The microbial composition of Murcha comprises a diverse range of yeasts and bacteria, with yeasts of genus *Saccharomycopsis* standing out as the predominant species which highlights their significance in the process of amylolytic fermentation (Hesseltine and Kurtzman, 1990; Tamang and Sarkar, 1996).

Yeast, a single-celled eukaryotic organism that belongs to the kingdom Fungi and reproduce through budding and they possess characteristics similar to those of eukaryotic cells found in plants and animals. The organisms like *Filobasidiella, Rhodotorula* are categorized into Basidiomycetes and organisms like *Saccharomyces, Candida* are categorized into Ascomycetes group. There are over 1500 species of yeast, with 80% of them having potential uses in biotechnology. One of the most studied eukaryotic cell which is widely used in the biotechnology and food industry is *Saccharomyces cerevisiae*, belonging to the Ascomycetes phylum, is a well-known ellipsoidal shaped non-pathogenic yeast species having 5-10 mm diameter and can withstand high concentrations of ethanol and butanol. Numerous uses of *Saccharomyces cerevisiae* have also been established through the utilization of genetic and metabolic engineering techniques

(Turker, 2014; Hartwell, 1974; Legras et al., 2007; Shen et al., 2021; Zhong et al., 2021).

Numerous types of yeast strains can be discovered in habitats abundant in nutrients, including fruits, tree bark, and soils (Alfenore et al., 2002). These microorganisms, which are more complex than bacteria, play an important role in various industrial applications. Yeast cells, characterized by their singular, oval shape, have approximate dimensions of 8 µm in length and 5 µm in diameter, setting them apart from other fungi and under ideal circumstances, yeast exhibits a rapid growth rate, doubling in population every 1-3 hours (Morris et al., 1992). Yeasts find widespread application in various industrial processes, including baking, brewing, and the production of diverse fermented products such as enzymes, vitamins, capsular polysaccharides, carotenoids, polyhydric alcohols, lipids, glycolipids, and citric acid, among others. Also, yeasts are used as a eukaryotic system to produce novel compounds. Extensive efforts have been undertaken to identify, document, and conserve yeast strains on a global scale due to their wide-ranging utilization and significance, as highlighted by James et al. in 1995.

One of the key characteristics of yeast cells that are significant both ecologically and in the industry is their resilience and ability to thrive in environments with high levels of ethanol. While ethanol can inhibit the growth of other microorganisms, excessive amounts can also cause stress on yeast cells. Various yeast strains display varying levels of tolerance to ethanol, with those more tolerant having an increased likelihood of survival compared to those that are less tolerant and these properties are useful for research purposes (Steensels and Verstrepe, 2014; Snoek et al., 2015; Mukherjee et al., 2014).

Ethanol can have a number of effects on cell growth and viability, including changes to the composition and structure of the cell membrane (Henderson

and Block, 2014). These changes can lead to an imbalance in the flow of ions, particularly protons, which can impair the ability of yeast cells to absorb nutrients. Additionally, ethanol can also impact the activity of important ions such as magnesium, which can play a role in both fermentation processes and in protecting cells from ethanol-induced stress. Ethyl alcohol reduces the activity of the semipermeable membrane H^+-ATPase (Aguilera et al., 2006) and of glycolytic enzymes because of reduced water activity. Research has also suggested that ethanol can lead to damage to the DNA in the cell's mitochondria, which can have further detrimental effects on cell growth and survival. Researchers have utilized DNA microarray techniques to examine the comprehensive gene expression patterns of yeast strains subjected to different levels of ethanol stress, such as 7% (Alexandre et al., 2001) and 5% (Hirasawa et al., 2007), in comparison to to those strains not exposed to such stress. Additionally, microarray techniques have been employed to investigate disparities in gene expression between ethanol-tolerant and ethanol-sensitive strains within comparable conditions (Deparis et al., 2017).

A technique that combines traditional fermentation with electrochemistry, called electro-fermentation technology, can be utilized to improve the production of ethanol. This novel process allows for the manipulation of microbial fermentative metabolism by means of electrochemical control. This approach offers several advantages, including enhanced efficiency in utilizing sugars, improved cell growth, decreased reliance on additives for redox balance or pH control, and, in certain instances, enhanced product recovery (Schievano et al., 2016). The electrodes employed within the electrochemical cell possess the ability to function as electron acceptors or donors, causing the polarization of ions in the microorganisms. This polarization resulting from electrochemical modifications disrupts the growth equilibrium, resulting in significant effects on metabolism, cellular regulation, interspecies interactions, and the selection of microbial populations (Moscoviz et al., 2016).

Scientists have utilized electrochemistry in their study of yeast. For example, Corton et al. (2001) discovered a way to directly identify specific microbial species, including *S. cerevisiae*, using a gold working electrode and cyclic voltammetry (CV) on cell culture samples without any pretreatment. Zhao et al. (2008) used platinum microelectrodes to investigate the effect of acetic acid on *S. cerevisiae*. Furthermore, Posseckardt et al. (2018) devised a technique employing electrochemical impedance microscopy with platinum screen-printing electrodes to assess cell viability, which demonstrated elevated measurements for viable cells. Similarly, Valiūnienė et al. (2020) established a protocol utilizing electrochemical impedance microscopy to differentiate between active and inactive cells of *S. cerevisiae*. Their observations indicated that the charge transfer resistance of active cells was 1.5 times lower compared to that of inactive yeast cells. This research focuses on effect of voltage on glucose utilization and ethanol production by yeast from Murcha of Nepal.

1.2. Research Hypothesis

1.2.1. Null Hypothesis

There is no significant difference in production of ethanol with or without supply of electricity.

1.2.2. Alternative Hypothesis

There is significant difference in production of electricity when yeast cells are supplied with electricity.

1.3. Objectives

1.3.1. General Objective

1. Isolation and characterization of salt and ethanol tolerant yeasts, to study their efficacy on electrochemical fermentation for ethanol production.

1.3.2. Specific Objective

1. Study on salt and ethanol tolerance of isolated yeasts.

2. Study on effects of different electrodes in glucose metabolism and ethanol production in yeast.

3. To study the effect of supplied voltage in glucose metabolism and ethanol production.

4.To compare performance based on glucose consumption and ethanol production of electrochemically treated yeast with the yeast grown in normal conditions.

1.4. Rationale of study

Murcha is a traditional starter culture used in Nepal for fermenting various food and beverage products. Understanding the growth characteristics of yeast isolated from murcha can provide valuable insights into their adaptation to these unique environmental conditions. Salt and ethanol tolerant yeast strains have significant industrial applications, especially in the production of fermented food and beverages. By studying the growth of these yeast strains using different electrodes, we can explore their potential

for microbial electrosynthesis and the production of biofuels or other value-added chemicals. This research can contribute to the development of sustainable and efficient industrial processes.

CHAPTER 2. LITERATURE REVIEW

2.1. Yeast

Yeasts are a type of microorganism that belong to the kingdom of fungi. *Saccharomyces cerevisiae* (*S. cerevisiae*), commonly known as Baker's yeast or Brewer's yeast, is a well-known species of yeast that is widely used in various industries and scientific research. It plays a crucial role in the production of wine, beer, and bread. In 1996, scientists successfully sequenced the entire genome of *S. cerevisiae* (Goffeau et al., 1996). During ethanol fermentation, *S. cerevisiae* utilizes sugars such as glucose, fructose, and sucrose to produce ATP (Adenosine triphosphate). As a result, ethanol and carbon dioxide are generated as byproducts. Furthermore, *S. cerevisiae* possesses the ability to ferment maltose, a process known as zymase and maltase activity of yeast (Griffin, 1969; White, 1956).

2.2. Yeast stress

Numerous yeast species possess the ability to convert diverse hexose sugars into ethanol via the process of glycolysis. Nevertheless, *Saccharomyces cerevisiae* stands out as the most commonly employed yeast organism for alcohol fermentation owing to its exceptional robustness and tolerance. This yeast is a facultative anaerobe, meaning it can grow in both the presence and absence of oxygen in the presence of glucose. It is also able to withstand high concentrations of ethanol. In the absence of oxygen, *S. cerevisiae* generates acetaldehyde, which subsequently undergoes conversion into ethanol (Claassen et al., 1999).

During the process of inoculating yeast cells and allowing them to ferment, a range of stress factors can impact the production of bioethanol. These stressors can be classified into biological, chemical, and physical stressors, such as toxicity arising from ethanol, cellular aging, competition from other microorganisms, and its byproducts, fluctuations in pH levels, sudden changes in temperature, and osmotic pressure (Walker et al., 2020). These

stressors can have negative effects on yeast cells through various mechanisms. They can lead to the introduction of microbial contamination, increased mutation rates, changes in yeast clumping behavior, reduced ethanol production, heightened glycerol production, and the formation of undesirable flavors and aromas in fermented beverages (Cray et al., 2015; Deparis et al., 2017).

In order to adapt to chemical and physical stress, yeast cells have developed various mechanisms. One such example is the production of the disaccharide trehalose in response to temperature stress, which helps to stabilize the plasma membrane (Cray et al., 2015; de Souza et al, 2018). Yeasts employ the production of glycerol as a protective mechanism to counter osmotic stress and prevent cell lysis when exposed to high-sugar or high-salt conditions (Andre et al., 1991; Larsoon et al., 1993; Ansell et al., 1997). Additionally, glycerol is generated to maintain a balanced NAD+/NADH ratio during cell growth (Nissen et al., 2000). However, the synthesis of these metabolites can impede the efficiency of ethanol production as it takes additional time for the cells to adapt to the environment associated to fermentation. To optimize ethanol yield, it is crucial to minimize the acclimation period by providing an optimal growth medium (Pagliardini et al., 2013).

2.2.1. Ethanol Tolerance of Yeast

The impact of ethanol on yeast growth varies depending on its concentration. When present at low levels, ethanol can hinder cell division and reduce the growth rate of cells, including their size and volume. However, at high concentrations, it can diminish cell vitality and lead to increased cell death (Birch and Walker, 2000). Moreover, ethanol can influence yeast cell metabolism and the biosynthesis of large molecules. It induces the production of proteins similar to those activated by heat shock, increases the frequency of petite mutations, denatures intracellular proteins and enzymes involved in glycolysis, reduces the accumulation of RNA and protein, and alters yeast's metabolism (Hu et al., 2007).

Yeast is commonly employed in ethanol production; however, it is sensitive to high concentrations of ethanol, particularly in conditions of very high gravity or high-gravity fermentation. As the concentration of ethanol increases in the fermentation medium, it can reduce the overall ethanol yield in the culture, hinder cell growth and viability, and disrupt transport systems (Casey and Ingledew, 1986; D'Amore et al., 1990; D'Amore and Stewart, 1987; Bai et al., 2004; Pina et al., 2004). Even at lower concentrations, ethanol can impede yeast growth by inhibiting cell division, reducing the specific growth rate, and decreasing cell volume (Birch and Walker, 2000). Elevated levels of ethanol can also compromise the cell membrane's integrity, impact the permeability of ions, and decrease the fluidity of the plasma membrane. These effects can result in acidification of the intracellular and vacuolar environments (Van Uden, 1985; Rosa and Sá-Correia, 1996; Salgueiro et al., 1988; Teixeira et al., 2009).

2.2.2 Salt Tolerance of yeast

The proper balance of alkali cations is crucial for the proper function of many organisms, including yeasts. In industrial fermentation processes such as baking, brewing, and winemaking, yeast cells may be exposed to stress. Improving yeast's tolerance to salt can benefit the production of yeast biomass as well as the baking industry (Attfield, 1997). The yeast *S. cerevisiae* has high levels of potassium (K+) and low levels of sodium (Na+) in its cytoplasm. Although K+ is not commonly found in many natural habitats of yeast, it plays a vital role in various cellular processes and is the most prevalent cation in *S. cerevisiae*. In contrast, Na+ is more prevalent in natural habitats but its accumulation inside cells can be harmful. The balance between K+ and Na+ is regulated by transporters that control the influx and efflux of cations (Bañuelos et al, 2002). Salt stress can dehydrate yeast cells, cause physiological and biochemical changes, and affect gene regulation (Mager and Siderius, 2002). Yeast cells can resist Na+ stress through ion homeostasis, which is achieved by ion transport and detoxifying mechanisms,

and through osmotic adjustment, which is accomplished by accumulating solutes inside the cell (Dhar et al., 2011; François et al., 2012).

Exposure of yeast to high salt concentrations is the third most studied stress after heat shock. Studies have shown that when *S. cerevisiae* is heat shocked and then exposed to salt stress by being plated on agar containing 1.5 M NaCl, there is no increase in the plate count on the salt stress agar after heat shock, indicating that heat shock does not protect cells from salt stress (Trollmo et al., 1988). Another study found that when yeast cells were subjected to heat shock and then tested for salt tolerance by measuring methionine uptake under salt stress conditions, the uptake of methionine in cells exposed to salt stress did not recover more quickly after heat shock than in control cells, suggesting that heat shock does not protect cells from salt stress (Varela et al., 1992). These findings indicate that heat shock does not induce salt tolerance in *S. cerevisiae*.

Salt tolerance assays are simple, quick, and inexpensive, and can be easily performed in both laboratory and industrial settings. For example, a commonly used method is the spot test, where yeast cells are spotted onto agar plates containing increasing concentrations of salt, and the minimum concentration that allows cell growth is determined. (Varela et al., 2012). Salt tolerance assays only measure the tolerance of yeast to osmotic stress and do not provide information on other important factors that can affect yeast performance, such as pH, temperature, nutrient availability, and oxygen supply. Therefore, salt tolerance assays should be used in combination with other methods to fully evaluate yeast performance under actual fermentation conditions. (Bely et al., 2008). However salt tolerance assays may not always be a reliable predictor of yeast performance under actual fermentation conditions, as other factors such as the interaction between yeast and other microorganisms, the presence of inhibitors, and the dynamics of fermentation may also come into play. Therefore, the use of salt tolerance assays as a sole criterion for strain selection or optimization of fermentation conditions should be done with caution. (Varela et al., 2012)

2.3. Quantitative analysis of reducing sugars

Miller first introduced the DNS (3,5-dinitrosalicylic acid) method, which has since become widely utilized for measuring the concentration of reducing sugars in various contexts (Miller, 1959). In some cases, modified DNS reagents lacking phenol have been employed by researchers (Vermelho and Couri, 2013). As shown in **Figure 1**, the DNS method is based on the reaction between reducing sugars and DNS to produce gluconic acid and ANS (3-Amino-5-nitrosalicylic acid), which results in a color change from yellow-orange to red-brown. Simultaneously, there is an increase in absorbance at a wavelength of 540 nm, situated on the shoulder of the DNS absorbance peak (around 479 nm). To determine the quantity of reducing sugars present, a calibration curve can be constructed using standard solutions of a reducing sugar, followed by measuring the rise in absorbance at 540 nm.

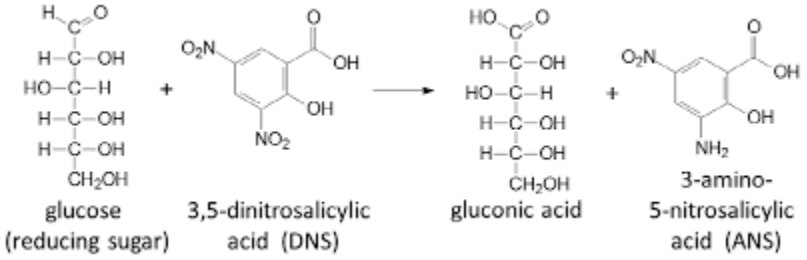

Figure 1: Representation of reaction between glucose as reducing sugar and DNS.

2.4. Quantitative analysis of ethanol

There are several techniques that can be used to measure the concentration of ethanol in an aqueous solution (Dubowski, 1980). One of the methods being gas chromatography (GC) remains the most commonly employed technique for ethanol analysis in alcoholic beverages. However, this method requires distillation, which can be costly, time-consuming, and labor-intensive (Santos et al., 2006; Lee et al., 2009). An alternative method that is simpler and more practical for measuring ethanol in various industries and research fields is solvent extraction and dichromate oxidation.

During fermentation, culture broth contains various complex polysaccharides, such as starch and cellulose. The enzymatic breakdown of these sugars during

saccharification can produce additional byproducts that can interfere with the color of the dichromate reagent used in ethanol measurement (Vincente et al., 2006; Archer et al., 2007). To avoid this interference, it is important to extract ethanol from the culture broth before measuring it. A method was developed for estimating bioethanol concentration in any aqueous solution using solvent extraction (Miah et al., 2017). Miah's study provides compelling evidence that this cost-effective solvent extraction method can be readily utilized to measure the concentration of ethanol in any aqueous solution.

Numerous solvents have been employed in the past to selectively extract ethanol, particularly from fermentation reactions (Alfenore et al., 2004). In the case of culture broths containing yeasts such as *Saccharomyces cerevisiae*, *Candida shehatae*, *Pichia kudriavzevii*, and *Candida tropicalis*, researchers have reported the successful utilization of the dichromate oxidation method for directly measuring ethanol concentration (Isarankura et al., 2007; Talukder et al., 2016). In this method, the presence of ethanol in an aqueous solution causes a color change from orange to blue-green as chromium ions oxidize the ethanol, leading to the reduction of the ions from their +6 oxidation state to +3. Other solvents, such as aliphatic alcohols like n-dodecanol or n-decanol, have also been employed for ethanol extraction from culture broths, while benzyl alcohol has served as an alternative to distillation (Cardona et al., 2007; Minier et al., 1982). Additionally, Tri-n-butyl phosphate (TBP), a non-alcoholic solvent, has been utilized to extract ethanol from culture broths due to the efficient oxidation of alcoholic solvents by the dichromate solution (Miah et al., 2017).

2.5. Electrochemical cell

Bioelectrochemical systems (BESs), including microbial electrolysis cells (MECs) (Liu et al., 2005) and microbial fuel cells (MFCs) (Santoro et al., 2017), are emerging technologies capable of harnessing the potential chemical energy found in organic compounds. Both MFCs and MECs exhibit similar anodic reactions, enabling the utilization of various organic sources present in wastewater, such as carbohydrates and lipids, as carbon sources for

bacterial activity at the anode (Rivera et al., 2017; Friman et al., 2017). Bacterial activity at the anode in both MFCs and MECs generates electrons and protons. The primary distinction between these technologies lies in the cathode activity and operational mode. In MFCs, electrical current is spontaneously generated due to the presence of an oxidizing agent (typically oxygen) (Fonseca et al., 2021; Hoang et al., 2022). Conversely, MECs require a specific amount of electrical input (around 0.3–0.8 V) to drive the redox reactions (Lim et al., 2019; Lim et al., 2021; Wang et al., 2021). The outputs of MFCs and MECs are electricity and hydrogen, respectively. Therefore, both MECs and MFCs offer the ability to convert waste streams with negative value into valuable products.

An external voltage has the capability to polarize the ions present in the cytosol, facilitating the transfer of electrons from the cathode to positively charged NAD+ ions bound to the cell membrane. This process is known to be accelerated, enabling the easier reduction of NAD+ to NADH in the cathode, ultimately leading to an increase in ethanol production (Gunawardena et al., 2008; McGillivray and Gow, 2009). Yau et al. (2013) propose that the application of an external voltage can potentially polarize the ionic charges within yeast cells and this polarization effect may subsequently reduce the tunnel barrier for electron transfer during glucose oxidation. As a result, more pyruvate is produced, promoting enhanced cell growth and increased ethanol production.

During glycolysis, pyruvate is produced, and its fate depends on the specific conditions and organisms involved. Under aerobic conditions in eukaryotes, pyruvate can be converted to acetyl CoA. In mammals during homolactic fermentation, pyruvate is converted to lactate. In yeast and bacteria during anaerobic fermentation, pyruvate is converted to ethanol. When an external voltage is applied, NAD+ is directly converted to NADH, leading to an increase in NADH levels. This imbalance can influence yeast growth and promote ethanol production by stimulating the conversion of pyruvate to acetaldehyde and then to ethanol using NADH. Elevated NADH levels also have an allosteric inhibitory effect on pyruvate dehydrogenase (Harris et al., 2002), preventing the conversion of pyruvate to acetyl coenzyme A and redirecting it towards ethanol formation. In the case of *Saccharomyces*

cerevisiae, the application of an external voltage enables the control of glucose metabolism in both aerobic and anaerobic conditions. Intracellular electron carriers such as NAD+, NADH, and the trans-plasma membrane electron transfer (tMPET) system in the plasma membrane play a crucial role in facilitating direct electron transport through the cell membrane. The tMPET system encompasses cytochromes and various redox enzymes, including NADH oxidase, which provides redox activity to specific sites in the membrane (Song et al., 2014).

2.5. PCR

The traditional approach to characterizing yeast biochemically involves conducting approximately 60-90 tests to ensure accurate species identification. This method is known for being intricate, demanding, and time-consuming (Deák, 1995). In contrast, molecular techniques offer a faster, simpler, and more precise means of yeast identification. These methods eliminate some of the subjectivity often associated with the results obtained from biochemical tests.

The polymerase chain reaction (PCR) method, developed by Kary Banks Mullis in 1983, is widely used in molecular biology to amplify specific DNA fragments from small amounts of source DNA or to amplify specific DNA fragments from RNA through a process called reverse transcription to create cDNA. (Saiki et al., 1985; Kyle and Shampo, 2002) The standard PCR reaction requires essential components such as thermostable polymerases (such as Taq), template DNA, primers, dNTPs, $MgCl_2$, and lab equipment such as autopipettes, plasticware, gloves, and a thermal cycler.

The PCR method amplifies specific DNA segments by repeatedly heating and cooling the target DNA molecule. At high temperatures near 95°C, the bonds between the A-T and G-C base pairs that hold the DNA's double helix structure break, separating the strands. Specific primers, which are complementary to the ends of the target DNA segment, bind to the separated strands at a lower temperature (50-65°C). The enzyme Taq polymerase then adds new nucleotides to the primers, forming a new copy of the target DNA segment at 72°C by adding dNTPs. This process is repeated

multiple times to generate many copies of the target DNA. It is important to follow best practice guidelines, such as those from the European Molecular Genetics Quality Network (EMQN), for optimal results. (Source: Muller, 2001)

The identification of yeasts involves analyzing the sequences of ribosomal RNA, which are conserved. By studying the genes that code for both 18s and 26s RNA, researchers have found that these genes are not only useful for understanding the evolutionary relationships between organisms, but also for characterizing yeasts at the molecular level (Ciardo et al., 2006). However, early studies on yeast classification have revealed a discrepancy between the observed physical characteristics (phenotypes) and the genetic makeup (genotypes) of these organisms. Researchers have discovered that the most efficient approach for classifying yeasts in a coherent and systematic manner is to examine specific genes. These genes include those encoding the 18S rRNA (James et al., 1995), the DNA sequences pertaining to domains 1 and 2 of 26S rRNA (Kurtzman et al., 2015), and the internal transcribed spacer (ITS) of 18S rRNA (Ciardo et al., 2006). By analyzing these genetic regions, scientists can successfully classify yeasts with accuracy and precision.

2.5.1. Molecular analysis by PCR

Colony PCR is a widely used molecular biology technique that involves the amplification of DNA fragments from bacterial colonies. The technique is fast, efficient, and relatively simple, making it a popular choice for many researchers. The principle of colony PCR involves the amplification of DNA directly from bacterial colonies, without the need for DNA extraction or purification. Typically, a small amount of bacterial colony is added directly to the PCR reaction mix, which contains primers specific to the target DNA fragment. The PCR reaction is then performed using standard protocols, and the resulting amplification products can be analyzed by gel electrophoresis or other techniques to confirm the presence or absence of the target sequence.

This technique has several advantages over traditional methods, including speed, ease of use, and reduced risk of contamination. However, there are also some limitations to colony PCR, including the risk of false positives and limited sensitivity. Despite these limitations, colony PCR remains a widely used technique in molecular biology and microbiology research. It has been

applied in a variety of contexts, including the identification of recombinant clones (Nour-Eldin et al., 2006), screening of microbial communities (Sakamoto et al., 2004), and detection of bacterial pathogens (Tanaka et al., 2007).

Numerous studies have demonstrated the usefulness of colony PCR in various applications. For example, Hagemann et al. (2014) used colony PCR to screen for antibiotic-resistant genes in bacteria isolated from wastewater. Their results showed that the method was effective in detecting a wide range of resistance genes, including those that are not commonly tested in clinical settings.

Another study by Dahle and colleagues (2015) utilized colony PCR to screen for the presence of a fungal endophyte in a plant species. The authors found that the technique was successful in detecting the fungal DNA in the plant tissue, demonstrating its potential for use in ecological and environmental research.

2.6. Bioreactor

A bioreactor is a device designed to facilitate chemical transformations by utilizing biological catalysts. It provides a controlled environment for efficient transfer of nutrients, metabolic products, and oxygen to and from cells (Sharma K.R, 2012; El AJ Haj et al., 2005; Bueno E.M et al., 2004). Bioreactor can accommodate various types of raw materials, including organic and inorganic compounds or complex substances. It has the capability to produce a wide range of products such as Baker's yeast, starter cultures, single-cell protein, animal feed, primary metabolites like amino acids, organic acids, and vitamins, as well as secondary metabolites including antibiotics. Bioreactors serve multiple applications, including bioconversion and biotransformation of products such as L-sorbitol, enzyme production, and the production of recombinant products like vaccines and hormones. Different bioreactor designs have been developed to cater to the specific requirements of different substrates and biocatalysts (ncsu.edu/biosuceed/courses).

Bioreactors are available in various sizes, encompassing a broad range of capacities. These sizes can range from small microbial cell cultures measured in cubic meters (m^3) to shake flasks with a capacity of 100-1000 mL. Other sizes include laboratory-scale fermenters ranging from 1 to 50 L, pilot-scale systems with capacities of 0.3-10 m^3, and large-scale industrial systems utilized for high-volume applications ranging from 2 to 500 m^3 (ncsu.edu/biosucceed/courses). The fundamental components of a bioreactor comprise a agitator system, headspace volume, oxygen delivery system, temperature and pH control system, foam control mechanism, sampling ports, cleaning and sterilization system, as well as charging and emptying lines for the reactor (Alaghlavi, 2013).

All the major components of a bioreactor are briefly described below:

2.6.1. Headspace volume:

The headspace in a bioreactor refers to the portion of the total volume that remains unoccupied by the medium, microbes, and gas bubbles. The remaining volume, where these components are present, is known as the working volume. Generally, the working volume of a bioreactor constitutes approximately 70-80% of the total volume. However, it is important to note that this percentage may vary depending on the rate of foam formation within the reactor (Van't R, 1991).

2.6.2. Agitator system

The agitator system plays a crucial role within the bioreactor and consists of key components such as an external power drive, baffles, and an impeller. Its primary function is to facilitate vigorous mixing and enhance the rate of mass transfer throughout the liquid and bubble layers. Additionally, the system generates the required shear forces to disperse and break up bubbles. Among the various impellers used in microbial fermentation, the Rushton turbine is widely employed as the preferred choice (srmuni.ac.in).

2.6.3. Air delivery system

The air delivery system of the bioreactor comprises several components, including a sterilization system, inlet air, compressor, air sparger, and an exit air sterilization system. These components work together to ensure the

delivery of clean and sterile air into the bioreactor, preventing any contamination.

2.6.4. Foam control system

The foam control system is an essential component aimed at mitigating the formation of excessive foam. Excessive foam can cause blockage in air exit filters and result in increased pressure within the reactor. Therefore, the foam control system plays a crucial role in maintaining optimal conditions within the bioreactor by effectively managing foam formation.

2.6.5. Temperature control system

The temperature control system of the bioreactor consists of temperature probes and a heat transfer mechanism, such as a jacket or coil. Heating is accomplished using electric heaters or steam generated in boilers, while cooling is achieved through the utilization of cooling water from cooling towers or refrigerants like ammonia. This system ensures precise temperature regulation within the bioreactor, allowing for optimal conditions for biological processes.

2.6.6. pH control system

The pH control system in the bioreactor employs neutralizing agents to maintain the desired pH level. It is crucial to select a non-corrosive and non-toxic neutralizing agent that does not harm the cells when diluted in the culture medium. Sodium carbonate is frequently utilized in small-scale bioreactors for this purpose.

2.6.7. Sampling ports

Sampling ports serve multiple purposes, including the injection of nutrients, water, and salts into the bioreactor, as well as the collection of samples for analysis or monitoring. These ports provide convenient access points for various operations related to nutrient supplementation, maintaining desired conditions, and obtaining representative samples from the bioreactor.

2.6.8. Cleaning and sterilization system

The cleaning and sterilization system plays a vital role in preventing contamination within the bioreactor. For large-scale equipment, thermal

sterilization is commonly preferred, while heat-sensitive equipment is typically subjected to chemical sterilization. Alternatively, sterilization can be achieved using radiation methods, such as UV radiation for surfaces and X-rays for liquids. Another approach involves the use of membrane filters with uniform microspores or depth filters containing materials like glass wool. These methods ensure effective sterilization, maintaining a sterile environment within the bioreactor (Van't R, 1991).

2.6.9. Charging and sampling lines

Charging and sampling lines serve the purpose of introducing reactants into the bioreactor and extracting products from it. These lines provide pathways for the controlled input of desired substances and the retrieval of the resulting products.

2.7. High Performance Liquid Chromatography (HPLC) for analysis of reactants and products

The term "chromatography" is derived from the Greek words "chroma," meaning color, and "graphein," meaning writing. High-performance liquid chromatography (HPLC) is a separation method that gets its name from the high pressure and is used to reduce analysis time. HPLC can be used to evaluate a wide range of compounds, from those with low molecular masses to those with extremely high molecular masses. The method uses two immiscible phases viz. a mobile phase and a stationary phase, to pass a dissolved mixture. The choice of liquid chromatography method depends on the nature of the analytes, their chemical composition, and molecular weight (Lozano et al., 2018).

In the early 21st century, advances in column technology and the use of adsorbents with smaller particle sizes (1.8 micrometers) in conjunction with newly developed instrumentation that allows the application of high pressure up to 1000 bar for solvent delivery, led to a new level of performance referred to as "ultra-performance liquid chromatography" (UPLC). This technology has improved sensitivity, speed, and resolution. (de Villiers et al., 2006; Cooper et al., 2007).

In modern times, continuous HPLC (High-Performance Liquid Chromatography) methods have become prevalent, enabling online detection of compounds following the separation process. HPLC employs a diverse range of detectors and is frequently combined with other detection techniques like ultraviolet-visible (UV-Vis), mass spectrometry (MS), nuclear magnetic resonance (NMR), and Fourier transform Raman (FTR) spectroscopy. This combined approach allows for the analysis of complex mixtures, facilitating the separation, identification, and quantification of substances (Lozano et al., 2018).

2.7.1. Instrumentation of HPLC

A state-of-the-art HPLC system is composed of various components, including a mobile phase delivery system, a column, a sample injector, a detection system, and a computerized data station.

2.7.1.1. Mobile Phase/ Solvent Reservoir

The mobile phase reservoir/container plays a critical role in an HPLC system as it holds the solvent utilized in the system. It is of utmost importance that the container is clean and chemically inert. Typically, the reservoir has a capacity of 0.5-2 L of solvent and is equipped with a cap that incorporates a tubing inlet line for supplying the mobile phase to the solvent delivery system. The cap on the container serves several functions, including preventing the entry of dust particles, reducing solvent evaporation, enabling pressurization, providing ports for additional inlet lines, and allowing for sparging. Sparging involves introducing gases such as helium or argon into the mobile phase to reduce the presence of dissolved air. Prior to use, it is crucial to filter and degas all mobile phases and solvents. In-line degassers have become increasingly common in many HPLC systems, as they effectively eliminate minute gas bubbles and minimize dissolved air. By incorporating these degassers, there is no longer a need to degas the mobile phase offline (LaCourse and LaCourse, 2017).

2.7.1.2. Solvent Delivery System

The high-pressure pump plays a crucial role in an HPLC system by delivering a constant, precise, and dependable flow of the mobile phase to the column.

This component ensures the consistent movement of the mobile phase, enabling accurate and reliable separation and analysis within the HPLC system. The pump is capable of operating at pressures ranging from 500 to 20,000 psi and ensures that the flow is precise, reproducible, constant and free from pulses. This allows for efficient and effective separation of the sample components in the column (LaCourse and LaCourse, 2017).

2.7.1.3. Sample Introduction Device

The introduction of the sample into the HPLC system is facilitated by a sample introduction device, commonly referred to as a sample injector. This device allows for the introduction of the sample into the system without the need for depressurization. The most prevalent method for sample injection involves the use of a sample loop that can be inserted or withdrawn from the mobile phase flow path by manipulating a valve. Automation of this process can be achieved using robotic technology to control the valve-based sample injection technique. Autoinjectors or autosamplers are increasingly utilized in HPLC systems to enhance injection reproducibility and enable the simultaneous handling of multiple samples. These autoinjectors incorporate an XYZ positioning device that directs a syringe-pump-driven needle to different positions containing samples. The samples can be stored in well plates or vials housed in sample racks at specific positions. The autoinjector is pre-programmed to withdraw a designated volume of sample from the specified location (LaCourse and LaCourse, 2017).

2.7.1.4. Column

The automation of the valve-based sample introduction system in HPLC can be achieved through the implementation of robotic technology. To optimize performance for specific analytes, different materials such as titanium, heavy-walled glass, and plastic (such as PEEK) can be employed. Analytical scale columns utilized in HPLC typically have dimensions ranging from 10 to 30 cm in length and 4 to 10 mm in diameter. The packing materials used in HPLC columns commonly have particle sizes of 3, 5, and 10 µm. Packing materials used in HPLC columns can be classified based on various factors, including rigidity, shape, and surface properties such as porosity. It is important to note that the properties of the packing materials may vary

depending on the specific chromatographic technique employed. Silica gel is the most commonly utilized adsorbent in liquid chromatography, as it possesses a range of characteristics that can be effectively utilized in HPLC (LaCourse and LaCourse, 2017).

2.7.1.5. Detector

In HPLC, the effluent from the column is directed to a detector, which transforms a specific chemical or physical property of the analyte into an electrical signal. This signal serves to monitor the eluted solutes as they pass through the column. The electrical signal generated by the detector can be amplified and manipulated using appropriate electronics, and its magnitude correlates with certain characteristics of the mobile phase or solutes. A range of detector types are employed in HPLC, including UV-visible detectors, fluorescence detectors, electrochemical detectors, mass spectrometry, conductivity detectors, and refractive index detectors. Each detector operates based on different principles and allows for the detection and analysis of specific properties or components within the eluted solutes (LaCourse and LaCourse, 2017).

2.7.1.6. Data Collection and Output

An electronic output device, such as a computer, integrator, or recorder, along with a data collection device, is linked to the electronic output of the detector. This setup allows the device to receive the electronic signal generated by the detector and generate a graphical representation of the signal's response over time. This graphical representation is commonly referred to as a chromatogram. The chromatogram provides valuable information that can be analyzed both qualitatively and quantitatively, offering insights into the composition and characteristics of the sample under examination (LaCourse and LaCourse, 2017).

CHAPTER 3. METHODS AND METHODOLOGY

3.1. Sample collection

The murcha samples were collected from different areas of Nepal i.e. Lubhu, Bhaktapur, Bhojpur and Satungal (**Figure 1**). They were given name (**Table 1**), then kept in sterile ziplock bag and stored at 4°C until further analysis.

Table 1: List of the collected murcha samples

S.N.	Name given to the sample	Color of the sample	Name of the district
1	Lubhu 1	White	Lalitpur
2.	Lubhu 3	White	
3.	Bhaktapur 1	Light yellow	Bhaktapur
4.	Bhaktapur 3	Light yellow	
5.	Bhaktapur 4	Light yellow	
6.	Bhojpur 1	White	Bhojpur
7.	Bhojpur 4	Creamy	
8.	Bhojpur 6	White	
9.	Satungal 5	Light yellow	Kathmandu
10.	Satungal 7	Light yellow	

Figure 1: GIS map of collection sites: Map shows the location of sample collection points Satungal, Lubhu, Bhaktapur and Bhojpur in reference to Kathmandu, Nepal. [*Source: Google Map, 2023*]

3.2. Isolation/ identification

The sample were serially diluted (10^{-1}-10^{-9}) and about 100 ul of diluted sample were pipetted on center of culture media and spread with spread evenly of surface of media. They were cultured on YEPD (Yeast extract peptone dextrose) agar media and incubated at 28°C for 2-3 days (Martins et al., 2019). The colonies were selected on the basis of their shape, size, color, consistency etc. Inoculums were prepared by inoculating a loopful of agar culture of isolated strain on PYN (Peptone Yeast extract and Nutrient) media (Balakumar and Arasaratnam, 2012) supplemented with 6.5% glucose (Rahman et al., 2013; Shamim et al., 2016).

3.3. Ethanol and salt tolerance

Yeast isolates derived from murcha were subjected to tests to assess their tolerance to ethanol and salt. The yeast strain was introduced into YEPD broth containing varying concentrations of ethanol (5%, 10%, and 15% w/v) and salt (5%, 10%, 15%, and 20%). Following incubation overnight at 28°C, the viability of the yeast cells was evaluated by measuring the absorbance at 600 nm. Among the yeast isolates, the one displaying the highest tolerance to

both ethanol and salt was selected for further analysis and subsequent experiments (Thammasittirong et al., 2013; Jansen et al., 2003).

3.4. Construction of Electrochemical Cell

The cathodic and anodic chambers were tightly assembled using a rubber gasket, with a nafion membrane serving as a separator. The working volume of each chamber was approximately 300 mL. For the cathode, a normal graphite felt measuring 11 cm * 3 cm was employed, while the anode consisted of platinum wire with a diameter of 0.2 mm and a length of 1 m. Each time, 0.4% of inoculum prepared was added to 300 mL of PYN media in each compartment (Joshi et al., 2019).

3.5. Ethanol production under applied voltage

Production of ethanol was monitored in cathodic and anodic compartments by culturing yeast strains in various combinations as follows (**Table 2**):

Table 2: Setup for different fermentation reaction. Four different setup for fermentation reaction was used.

Fermentation reaction setup	**Anode**	**Cathode**
Setup 1 (G-P)	Graphite	Platinum
Setup 2 (G-G)	Graphite	Graphite
Setup 3	Graphite	Graphite with ferricyanide @ 0.2 mg/mL
Setup 4	Graphite	Graphite with a nitrogen purge of

		2 minutes

In the first fermentation reaction, the yeast strains were cultured in either chambers using graphite as anode and platinum as cathode. In reaction 2 graphite was used both as anode and cathode. In reaction 3, graphite-graphite electrode was used, where cathode was treated with ferricyanide. In reaction 4, graphite-graphite electrode was used, with supply of nitrogen gas at cathode. External voltage 4V was applied (Joshi et al., 2019).

The reducing sugar of sample was measured by Dinitrosalicylic Acid (DNSA) method, and the concentration of ethanol sample was measurerd by Tri-n-butyl phosphate (TBP) method. They were compared with sample that was incubated at 28°C with and without shaking.

3.6. Determination of reducing sugars by DNSA

One mL of sample was centrifuged at 12000 rpm for 5 mins. The supernatant was mixed with 3mL DNSA reagent (Appendix III). They were heated in boiling water bath for 10 minutes and left to cool for 10-20 minutes. The intensity of color change was measured at 540 nm at stipulated time intervals (24, 48, 72, 96 and 120 hrs) for the estimation of reducing sugar during the growth of yeast cells. The concentration of glucose was measured by comparing it with standard curve of glucose. (Abd-Rahim et al., 2014)

3.7. Ethanol determination by Tri-n butyl phosphate (TBP) test

One mL of the yeast culture was subjected to centrifugation at 4000 g for 15 minutes. The resulting supernatant was carefully transferred to a clean Eppendorf tube. An equal volume of TBP (Tri-n-butyl phosphate) was added to the supernatant, followed by vortexing for approximately 10 minutes.

Subsequently, the mixture was centrifuged at 12000 g for 15 minutes. The upper layer of TBP, measuring approximately 750 µL, was then transferred to a clean tube. To this, an equal amount of acidified 5% potassium dichromate was added. The resulting mixture was vortexed and subjected to centrifugation at 4000 g to separate the layers. The green layer at the bottom was carefully extracted, and the absorbance was measured at 595 nm (Seo et al., 2009; Miah et al., 2017).

3.8. Determination of ethanol and glucose by HPLC

The sample of yeast was centrifuged at 12000 rpm for 10 minutes and was syringe filtered in HPLC vials. H_2SO_4 was used as the mobile phase.

The program for HPLC using Aminex HPX 87H column was set up as follows:

Column temp: 55°C

Injection volume: 10µl

H_2SO_4: 50 mM

Flow rate: 0.6 mL/min

Detector: RI

The ethanol and glucose concentration were determined by analysing RID (Refractive Index Detector) signals. The column was washed with 75 % acetonitrile for about 30 minutes. (Blake et al., 1987).

3.9. Polymerase chain Reaction (PCR)

The yeast isolates were grown on PYN agar media and sent to RIBB (Research Institute for Bioscience and Biotechnology) for colony PCR. A 18s rRNA primer (BioRad) was used for yeast identification.

Primer sequence: Forward primer: 5’ GGT CTT GTA ATT GGA ATG AG 3’

Reverse Primer: 5’ CTT CCG TCA ATT CTT TAA G 3’

The PCR condition for the organism are given below in **Table 3**:

Table 3: PCR Cycling Condition

	Steps	Temperature (°C)	Time (min)
Step 1	Initial Denaturation	95°C	2:00
Step 2	Denaturation	95°C	0:30
Step 3	Annealing	49°C	0:30
Step 4	Extension	72°C	1:10
Step 5	Repeat step 2-4, 29 cycles		
Step 6	Final extension	72°C	5:00
Step 7	Hold	12°C	2:00

The 18s rRNA PCR product was identified by agarose gel electrophoresis (Du et al., 2006). The sample was run on 0.8 % agarose gel having EtBr concentration of 50 µg/mL. 3 µl of PCR product were loaded on well along with positive control, negative control and ladder.

3.10. Protein Determination

Determination of ürotein content was done by the SDS-PAGE technique (Simpson RJ, 2006). The proteins were visualized by staining the gels with Coommassie Brilliant Blue. The resolving gel was prepared by mixing components (Appendix IV). It was cast after proper mixing upto level of the fill line. The top layer was overlaid with help of isopropanol and vertically left for about 10 minutes for polymerization. After polymerization of the resolving gel, the overlay iso-propanol was poured off, and the remaining was

wicked out with a piece of blotting paper. The stacking gel was prepared and immediately poured over resolving gel and comb was inserted slowly. It was then allowed to polymerize for 10 min. The polymerization was verified by examining leftover acrylamide on the tube. After polymerization, the comb was carefully removed. The slab was mounted in electrophoresis device, which was filled with running buffer in both tanks.

Protein samples of 20µl were added with 20µl loading buffer in an Eppendorf tube. The mixture was heated at 95°C for 5 minutes to denature the protein. Prepared protein samples and protein marker (11 to 250 kDa) were loaded in the wells in an order determined already. The SDS-PAGE was run with a constant voltage of 120V till the dye front reaches almost lower end of gel. After running, the gel was stained with staining solution (Appendix IV) for 1 hour, followed by destaining solution II (Appendix IV) overnight with destaining solution II changed intermittently (Manns et al., 2011).

3.11. Fermentation scale up by using bioreactor

Batch fermentation was carried out in 3-liter volume (Electrolab). A 10mL of prepared inoculum was poured on a bioreactor filled with PYN media. For yeast culture, the culture conditions were: temperature 28°C, pH 6.5, agitation 100 rpm. Sampling was done every 24 hours for 5 days. The glucose concentration was determined by DNSA test and ethanol concentration was determined by the TBP test.

3.12. Statistical analysis

GraphPad Prism 9.0 was used for the preparation of graphs and statistical analysis. Mean of triplicate readings were taken with standard deviation for all the data collected for experiment.

CHAPTER 4. RESULTS AND DISCUSSION

4.1. Isolation, identification and selection of yeasts from Murcha samples

Isolation, identification and selection of yeasts from Murcha samples were done. The details of the results are summarized below.

4.1.1 Isolation of yeast Murcha samples

Ten different samples of murcha were collected from five different places of Nepal out of Lubhu, Bhaktapur, Bhojpur and Satungal.

4.1.2. Sample selection and study of morphology

Growth characters of the yeast isolates were observed morphologically and microscopically after growing on liquid and solid YEPD media. As shown in **Figure 1,** creamy-colored, rounded, smooth margined with raised elevated yeast colonies having about 2 mm diameter were observed and these colonies from each sample were processed further. Validation was done with general microscopy as shown in **Figure 2**.

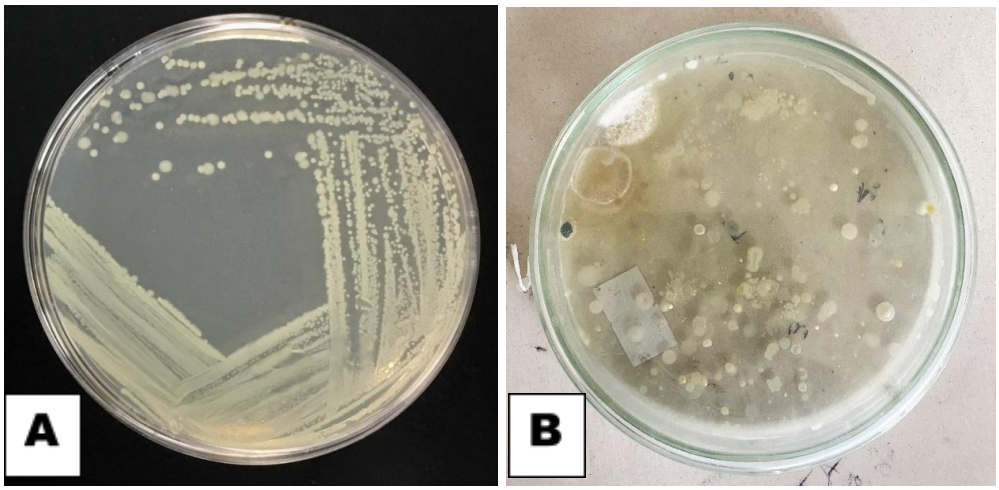

Figure 1: A. Colonies in YEPD agar media. B. Pure isolates of yeast in YEPD agar media. Round, creamy, smooth-edged colonies form the sample Bhojpur 4 were observed in plate A of Bhojpur 4. Plate B represents pure isolates selected from the plate A which was quadrant streaked using YEPD agar.

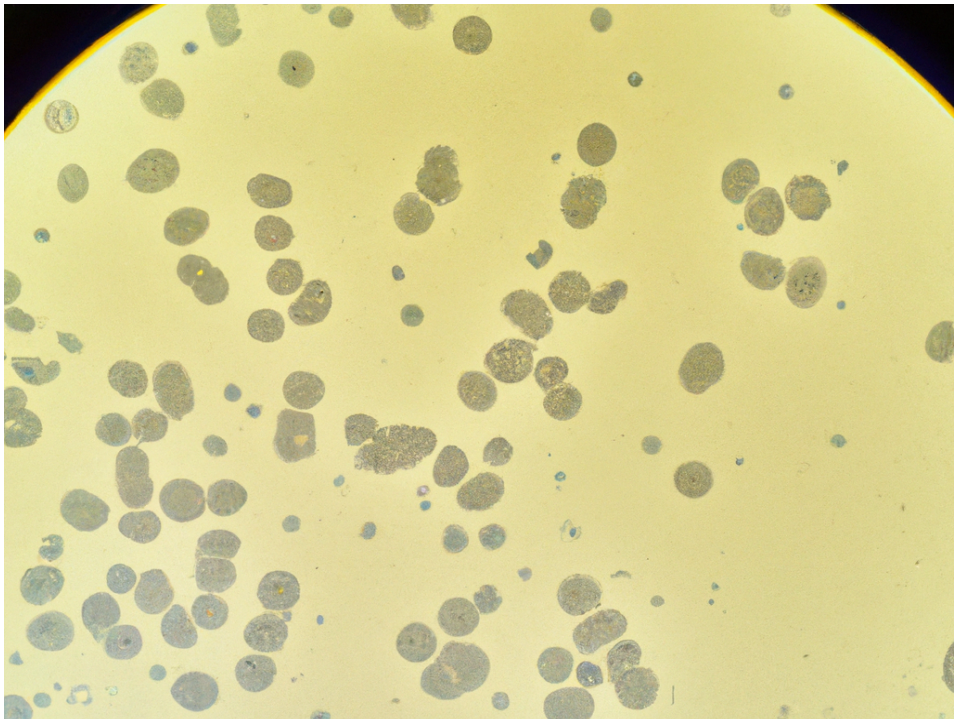

Figure 2: Microscopy of selected colony. The selected colony from Bhojpur 4 was observed under 40X without staining.

Several yeast strains were isolated from the murcha by various research groups worldwide (Tsuyoshi et al., 2005; Shankar et al., 2016; Sha et al. 2017). The yeast was also isolated from the murcha of Nepal collected from the four districts of Nepal viz. Lalitpur, Bhaktapur, Bhojpur and Kathmandu. Various salt-tolerant halophilic yeast strains were also identified by the researchers as is believed that these strains can adapt and tolerate extreme conditions (Ahangangoda et al. 2019; Musa et al. 2018). On the other hand, ethanol-tolerant yeast strains were also identified and isolated by various researchers as these strains are required to achieve high fermentation potential in order to produce biofuel from various sugar sources (Tikka et al., 2013; Villarreal 2022). This research also isolated (**Figure 1**) and microscopically identified (**Figure 2**) the yeast from the samples of Bhojpur 4.

4.1.3. Study of effect of ethanol concentration on yeast colonies

Variable results in the growth of selected yeast samples at log phase were observed at 600 nm while accessing growth at different ethanol concentration (5%, 8% and 10%) on PYN media. Result is shown in **Figure 3**.

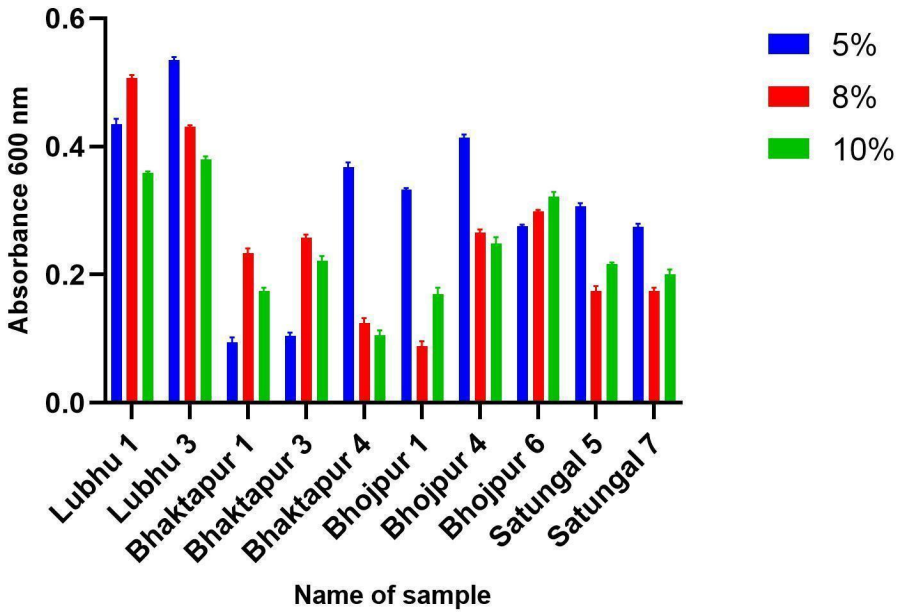

Figure 3: Growth pattern of different yeast samples at variable ethanol concentrations. The growth pattern observed in 5%, 8% and 10% ethanol concentrations using a UV spectrophotometer at 600 nm were recorded and absorbance values at the y-axis were plotted against the samples taken and ethanol concentrations in the x-axis. Triplicate data were taken to plot the graph using GraphPad Prism (n=3, $P<0.0001$).

From the graph shown in **Figure 3**, the highest growth at 5% ethanol concentration was observed in the yeast isolated from Lubhu 3 followed by Lubhu 1, Bhojpur 4, Bhaktapur 4 and Bhojpur 1. Similarly, the highest growth at 8% ethanol concentration was observed in the yeast isolated from Lubhu 1 followed by Lubhu 3, Bhojpur 6, Bhojpur 4 and Bhaktapur 3. Similarly, the

highest growth at 10% ethanol concentration was observed in the yeast isolated from Lubhu 3 followed by Lubhu 1, Bhojpur 6, Bhojpur 4 and Bhaktapur 3. Yeast isolated from samples Lubhu 3, Lubhu 1 and Bhojpur 4 showed wide and stable growth in the presence of ethanol among all the variations of ethanol concentrations.

The isolated sample from Bhojpur 4 was seen to have easily tolerated ethanol concentration from 5% to 10% as observed in **Figure 3**. The same yeast from the sample of Bhojpur 4 was further selected for experimentation. As observed in **Figure 3**, the variation or nonuniformity in the growth pattern seen in some of the yeast species at different concentrations of ethanol was reported by various researchers (Lee et al., 2011; Fakruddin et al., 2013). In general, most yeast species can tolerate ethanol concentrations of up to around 5-10% (v/v), which is the typical range found in many alcoholic beverages (Gao et al., 1988). However, some yeast species have been found to grow best at higher or lower ethanol concentrations, depending on their natural habitat and evolutionary history. Yeast grows best in certain ethanol concentrations because ethanol can either promote or inhibit their growth and metabolism, depending on the concentration and other environmental factors (Merico et al. 2007; Rozpędowska et al. 2011). Also, some of the yeast species were reported to have optimal growth at around 10% ethanol concentration (Yuangsaard et al., 2013). Thus, understanding the optimal ethanol concentration for a given yeast species can be important for optimizing fermentation processes and improving the efficiency of industrial applications.

4.1.4. Study of salt tolerance in yeast colonies

Variable results in the growth of isolated yeast samples were observed at 600 nm while accessing salt tolerance using PYN media as shown in **Figure 4**.

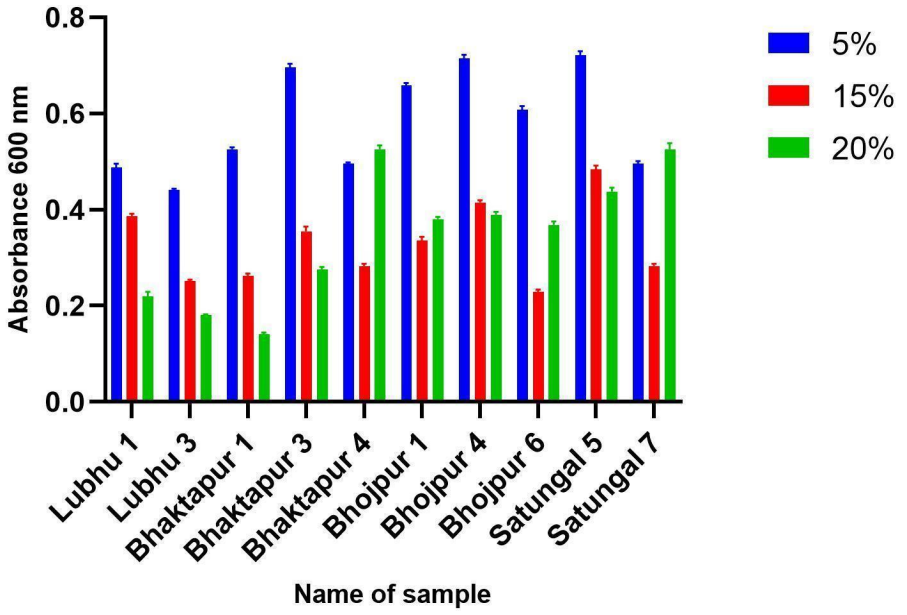

Figure 4: Growth pattern of different yeast samples at variable salt concentrations. The growth pattern observed in 5%, 15% and 20% salt concentrations using UV spectrophotometer at 600 nm were recorded and absorbance values at the y-axis were plotted against the samples taken and salt concentrations in the x-axis. Triplicate data were taken to plot the graph using GraphPad Prism (n=3, P<0.0001).

From the graph shown in **Figure 4**, the highest salt tolerance at 5% concentration was observed in the yeast isolated from Satungal 5 followed by Bhojpur 4, Bhaktapur 3, Bhojpur 1 and Bhojpur 6. Similarly, the highest salt tolerance at 15% concentration was observed in the yeast isolated from Satungal 5 followed by Bhojpur 4, Lubhu 1, Bhaktapur 3 and Bhojpur 1. Similarly, the highest salt tolerance at 20% concentration was observed in the yeast isolated from Bhaktapur 4 followed by Satungal 7, Satungal 5, Bhojpur 4 and Bhojpur 1. Yeast isolated from samples Satungal 5, Bhojpur 1 and Bhojpur 4 showed wide and stable tolerance among all the variations of salt concentrations.

By studying the data from both salt and ethanol tolerance, Bhojpur 4 exhibited wide and stable tolerance among all the ethanol and salt concentrations. Hence, the yeast isolated from Bhojpur 4 was selected for further experimentation. The isolated sample from Bhojpur 4 was seen to have easily tolerated salt concentration from 5% to 20% as observed in **Figure 4**.

Thus, the yeast from the sample of Bhojpur 4 was seen to have a wide range of ethanol (**Figure 3**) and salt (**Figure 4**) tolerance. These kind of salt and ethanol tolerant yeasts are thought to survive and perform best in microbial electrochemical cells having wider environmental conditions (Joshi et. al, 2019; Badalamenti et al. 2013; Stanley et al., 2010).

4.2. Study of the effect of electrochemical performance on the glucose metabolism of yeast

The standard glucose curve was constructed using DNSA method and a simple regression analysis was done (**Appendix I**). The linear regression analysis yielded an equation of Y=0.5121*X-0.004553.

Data from the standard glucose curve was used to plot **Table 1** showing the concentration of glucose in the different electrode setups.

Table 1: Effect of different electrochemical systems on the glucose metabolism of yeast. Two experimental setups viz. one setup called graphite-graphite (G-G) electrochemical cells where graphite was used as both anode and cathode and another setup called graphite-platinum (G-P) electrochemical cells where graphite was used as anode and platinum as a cathode are shown in the table. Yeast from the broth was added in both experimental setups and supplemented with 6.5% of glucose in the PYN media and 4V of electricity was supplied. The absorbances of glucose using DNSA at 540 nm were recorded for each experimental setup at the time

interval of 24 hrs, 48 hrs, 72 hrs, 96 hrs and 120 hrs and then the equation from linear regression analysis of the standard glucose curve was used to calculate the respective values of the glucose concentrations for each setup (n=3, P<0.05).

SN	**Sample**	**Time (hrs)**	**Mean glucose concentration (mg/mL) using DNSA in reference to the standard (±SD)**
	Graphite-Graphite electrochemical cell		
1	Anode (Graphite)	24	61.31 ± 0.23
		48	57.68 ± 0.25
		72	3.11 ± 0.08
		96	2.74 ± 0.10
		120	2.08 ± 0.03
2	Cathode (Graphite)	24	61.87 ± 0.30
		48	60.94 ± 0.49
		72	60.82 ± 0.43
		96	56.04 ± 0.22
		120	42.66 ± 0.15

	Graphite-Platinum electrochemical cell		
3	Anode (Graphite)	24	60.23 ± 0.52
		48	58.00 ± 0.25
		72	57.10 ± 0.22
		96	51.64 ± 0.12
		120	36.85 ± 0.61
4	Cathode (Platinum)	24	64.13 ± 0.14
		48	59.84 ± 0.10
		72	38.28 ± 0.13
		96	6.69 ± 0.15
		120	4.47 ± 0.15

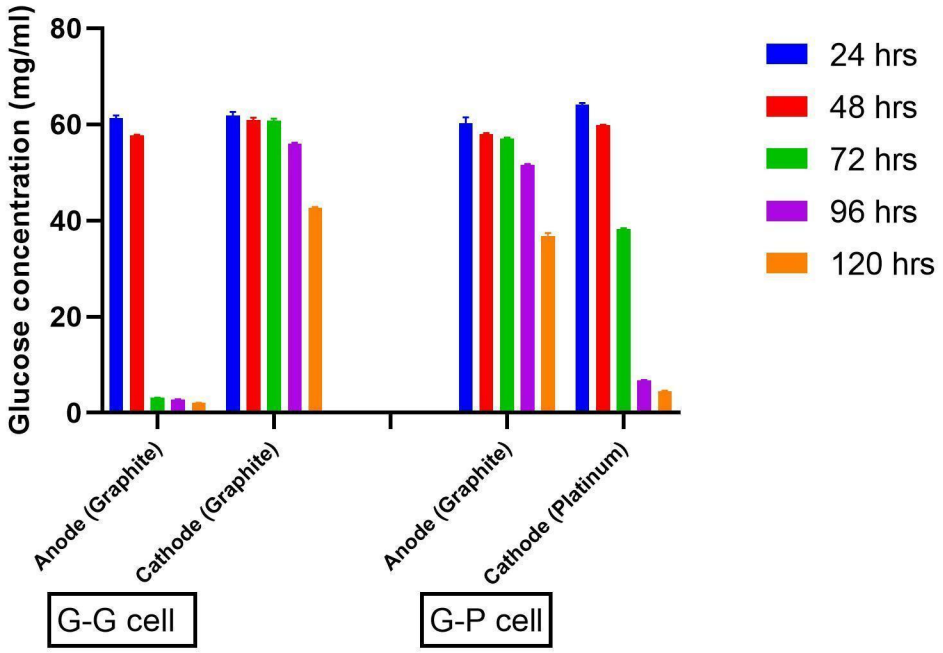

Figure 5: Comparative analysis between G-G and G-P electrochemical cells for glucose: The data from Table 2 were plotted keeping samples on the x-axis and the glucose concentration (mg/mL) on the y-axis. Time in hours is denoted in the bar. The G-G cell represents the graphite-graphite electrochemical cell and the G-P cell represents the graphite-platinum electrochemical cell each showing the respective anode and cathode.

The comparative graphical analysis of the data from **Table 1** was done in **Figure 5**. It was observed from **Table 1** and **Figure 5** that better activity of glucose metabolism of yeast was seen in the anode side using the graphite-graphite electrochemical cell than the graphite-platinum electrochemical cell. From **Table 1**, the glucose concentration in the anode side of the G-G cell was observed to be 3.11 ± 0.08 mg/mL at 72 hrs, 2.74 ± 0.10 mg/mL at 96 hrs, 2.08 ± 0.03 mg/mL at 120 hrs. The graphite-graphite electrochemical cell was selected further for experimentation.

Table 2: Effect of variable conditions on the glucose metabolism of yeast using graphite-graphite electrochemical cells. The table shows data of glucose concentration at different timeframes in the presence of yeast using the variations in the setup of graphite-graphite electrochemical cells viz. the first setup consists of the addition of ferricyanide in the graphite-graphite electrochemical cells at room temperature, second setup consists of the addition of 0.2 mg/mL of ferricyanide in the graphite-graphite electrochemical cells at 28^{o}C and the third setup consists of 2 minutes nitrogen purging at room temperature without ferricyanide treatment. All the experimental setups were supplemented with 6.5% of glucose in the PYN media and 4V of electricity was supplied. The absorbances of glucose using DNSA at 540 nm were recorded for each experimental setup at the time interval of 24 hrs, 48 hrs, 72 hrs, 96 hrs and 120 hrs and then the equation from linear regression analysis of the standard glucose curve was used to calculate the respective values of the glucose concentrations for each setup (n=3, P<0.05).

SN	**Sample**	**Time (hrs)**	**Mean glucose concentration (mg/mL) using DNSA in reference to the standard (± SD)**
	Graphite-Graphite with ferricyanide @ 0.2 mg/mL in 300 mL solution at room temperature		
1	Anode (Graphite)	24	60.28 ± 0.31
		48	60.12 ± 0.35
		72	59.81 ± 0.19
		96	59.74 ± 0.10

		120	59.15 ± 0.30
2	Cathode (Graphite)	24	60.58 ± 0.47
		48	60.24 ± 0.11
		72	59.78 ± 0.18
		96	59.76 ± 0.18
		120	59.19 ± 0.15
	Graphite-Graphite with ferricyanide @ 0.2 mg/mL in 300 mL solution at 28°C		
3	Anode (Graphite)	24	63.78 ± 0.49
		48	62.94 ± 0.42
		72	60.16 ± 0.50
		96	15.84 ± 0.22
		120	4.69 ± 0.13
4	Cathode (Graphite)	24	63.71 ± 0.56
		48	63.69 ± 0.45
		72	63.57 ± 0.46
		96	63.10 ± 0.46

		120	61.65 ± 0.54
	Graphite-Graphite with a nitrogen purge of 2 minutes		
5	Anode (Graphite)	24	60.65 ± 0.59
		48	59.76 ± 0.20
		72	50.47 ± 0.66
		96	3.67 ± 0.17
		120	1.21 ± 0.12
6	Cathode (Graphite)	24	62.62 ± 0.10
		48	62.48 ± 0.24
		72	62.42 ± 0.13
		96	62.39 ± 0.07
		120	62.29 ± 0.12

From **Table 2** it was observed that no significant change in the glucose concentration was seen when the ferricyanide treatment was done at room temperature. But when kept constantly at 28^{o}C which is the optimal temperature for the growth of yeast during the ferricyanide treatment, a significant increase in the metabolism of yeast was observed in the graphite anode at 120 hrs in. At 120 hrs, the glucose concentration dropped to 36.85 ± 0.61 mg/mL from the concentration of 51.64 ± 0.12 mg/mL at 72 hrs.

Similarly, from **Table 2** it was observed that without the ferricyanide treatment when a nitrogen purge was given at room temperature before the supply of electricity, then the optimal metabolism of the glucose in the yeast was seen at 96 hrs in the anode. At 96 hrs, the glucose concentration dropped to 3.67 ± 0.17 mg/mL from the concentration of 50.47 ± 0.66 mg/mL at 72 hrs. At 120 hrs the glucose concentration was 1.21 ± 0.12 mg/mL. As a negative control, experimental setup at 28^{o}C was carried out further without electricity.

Table 3: Scale-up of effect on the glucose metabolism of yeast without electricity, with shaking and using a bioreactor. The table represents data of glucose concentration at different timeframes in the presence of yeast and the absence of electricity. Data from three different setups are shown viz. first setup was carried out without electricity and shaking at 28^{o}C, the second setup was carried out without electricity but a shaking of 100 rpm was given at 28^{o}C and the third setup was carried out in a bioreactor giving temperature of 28^{o}C pH of 6.5 and agitation of 100 rpm. All the experimental setups were supplemented with 6.5% of glucose in the PYN media. The absorbances of glucose using DNSA at 540 nm were recorded for each experimental setup at the time interval of 24 hrs, 48 hrs, 72 hrs, 96 hrs and 120 hrs and then the equation from linear regression analysis of the standard glucose curve was used to calculate the respective values of the glucose concentrations for each setup ($n=3$, $P<0.05$).

SN	Sample	Time (hrs)	Mean glucose concentration (mg/mL) using DNSA in reference to the standard (±SD)
1	Without electricity and shaking at 28°C	24	63.57 ± 0.16
		48	63.39 ± 0.12
		72	41.99 ± 0.50
		96	22.44 ± 0.63
		120	5.57 ± 0.09
2	Without electricity and shaking at 100 rpm and 28°C	24	63.10 ± 0.57
		48	49.96 ± 0.09
		72	34.55 ± 0.30
		96	21.47 ± 0.32
		120	4.80 ± 0.08
3	3 liters culture in a bioreactor at pH 6.5, 100 rpm and 28°C	24	64.27 ± 0.55
		48	23.82 ± 0.16
		72	8.68 ± 0.13
		96	3.01 ± 0.04

		120	2.95 ± 0.04

The data from **Table 3** showed the highest metabolism of the glucose in the yeast in the bioreactor at 72 hrs. In the bioreactor, at 72 hrs, the glucose concentration dropped to 8.68 ± 0.13 mg/mL from the concentration of 23.82 ± 0.16 mg/mL at 72 hrs and at 96 hrs and 120 hrs the glucose concentration was 3.01 ± 0.04 mg/mL and 2.95 ± 0.04 mg/mL.

The second setup without the current supply where the shaking was done at 100 rpm at 28^{o}C showed the second highest metabolism of the glucose in the yeast at 96 hrs. In this second setup, at 96 hrs, the glucose concentration dropped to 21.47 ± 0.32 mg/mL from the concentration of 34.55 ± 0.30 mg/mL at 72 hrs and at 120 hrs the glucose concentration was 4.80 ± 0.08 mg/mL.

The first setup without the current and without shaking done at 100 rpm at 28^{o}C showed the least metabolism of the glucose in the yeast at 96 hrs. In this first setup, at 96 hrs, the glucose concentration dropped to 22.44 ± 0.63 mg/mL from the concentration of 41.99 ± 0.50 mg/mL at 72 hrs and at 120 hrs the glucose concentration was 5.57 ± 0.09 mg/mL.

4.3. Study of the effect of electrochemical cells on the ethanol production

The effect of different experimental setups on the ethanol production behavior of yeast is shown in the result. The standard ethanol curve was constructed using TBP method and a simple regression analysis was done (**Appendix II**). The linear regression analysis yielded an equation of Y = 0.03230*X + 0.05814.

Data from **the standard ethanol curve** was used to plot **Table 4** showing the concentration of ethanol in the different experimental setups.

Table 4: Effect of different electrochemical cells design on the ethanol production of yeast. Two experimental setups viz. one setup called graphite-graphite electrochemical cells where graphite was used as both anode and cathode and another setup called graphite-platinum electrochemical cells where graphite was used as anode and platinum as a cathode are shown in the table. Yeast from the broth was added in both experimental setups and supplemented with 6.5% of glucose in the PYN media and 4V of electricity was supplied. The absorbances of glucose using TBP method at 595 nm were recorded for each experimental setup at the time interval of 24 hrs, 48 hrs, 72 hrs, 96 hrs and 120 hrs and then the equation from linear regression analysis of the standard ethanol curve was used to calculate the respective values of the glucose concentrations for each setup (n=3, $P<0.05$).

SN	Sample	Time (hrs)	Mean ethanol concentration (mg/mL) using the method described by Seo at al., 2009 and Miah et al., 2017 in reference to the standard (±SD)	Percentage
	Graphite-Graphite (G-G) electrochemical cell			
1	Anode (Graphite)	24	14.23 ± 0.21	1.42
		48	28.76 ± 0.09	2.88
		72	51.76 ± 0.09	5.18

		96	57.74 ± 0.09	5.77
		120	29.28 ± 0.07	2.93
2	Cathode (Graphite)	24	7.83 ± 0.22	0.783
		48	21.82 ± 0.04	2.18
		72	23.06 ± 0.06	2.31
		96	32.29 ± 0.30	3.23
		120	39.87 ± 0.06	3.99
	Graphite-Platinum (G-P) electrochemical cell			
3	Anode (Graphite)	24	11.70 ± 0.10	1.17
		48	25.17 ± 0.06	2.52
		72	27.05 ± 0.06	2.71
		96	40.03 ± 0.07	4
		120	45.88 ± 0.06	4.59
4	Cathode (Platinum)	24	9.19 ± 0.07	0.92
		48	19.25 ± 0.09	1.93
		72	29.07 ± 0.08	2.91

		96	33.00 ± 0.05	3.3
		120	23.47 ± 0.05	2.35

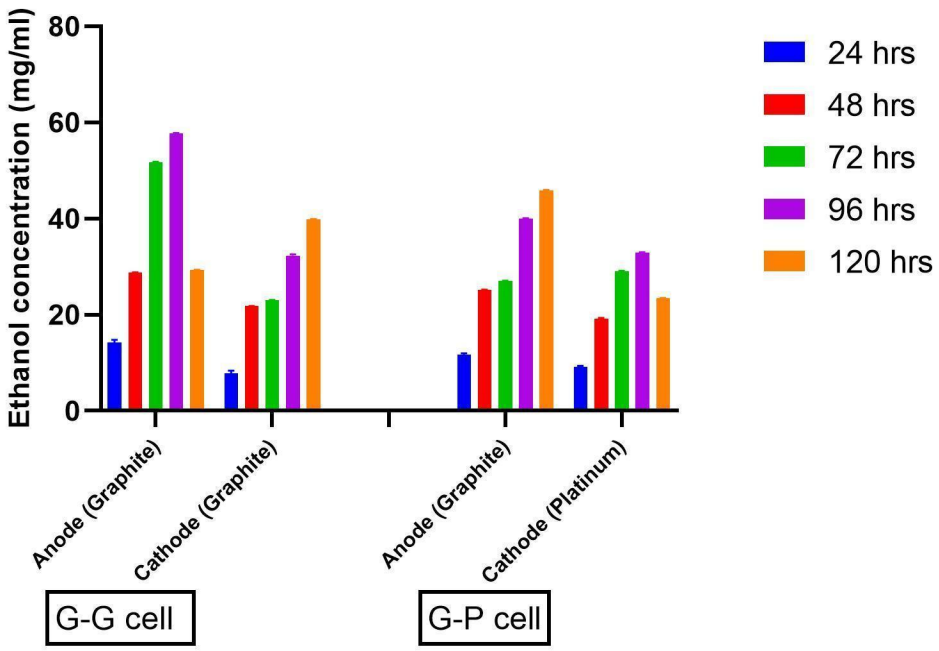

Figure 6: Comparative ethanol analysis between G-G and G-P electrochemical cells: The data from Table 5 were plotted keeping samples on the x-axis and the ethanol concentration on the y-axis. Time in hours is denoted in the bar. The G-G cell represents the graphite-graphite electrochemical cell and the G-P cell represents the graphite-platinum electrochemical cell each showing the respective anode and cathode for glucose utilization.

The comparative graphical analysis of the data from **Table 4** was done in **Figure 6**. It was observed from **Table 4** and **Figure 6** that better activity for ethanol production by yeast was seen in the anode side using the graphite-graphite electrochemical cell and in cathode side for the graphite-platinum electrochemical cell. From **Table 4**, the ethanol concentration in the anode side of the G-G cell was observed to be 51.76 ± 0.09 mg/mL at 72 hrs, 57.74 ± 0.09 mg/mL at 96 hrs, 29.28 ± 0.07 mg/mL at 120 hrs. The drop in the ethanol concentration was also noted in the graphite anode for graphite-graphite electrochemical cell setup at 120 hrs as compared to 96 hrs. The graphite-graphite electrochemical cell was selected further for experimentation.

From **Table 1**, **Table 4**, **Figure 5** and **Figure 6,** the metabolism of glucose and production of ethanol was seen in both graphite-graphite and graphite-platinum electrochemical cells and similar results were noted by other researchers as well (Chibueze et al., 2018; Yousefi et al., 2017; Tamakloe et al., 2015; Verma et al., 2021; Seuss 2013). The reason behind the low glucose metabolism activity in the graphite-platinum electrode can be due to the slight antifungal effect of platinum (Nam et.al., 2016; Jeyaraj et al., 2019).

Table 5: Effect of variable conditions on the ethanol production by yeast. The table shows data of ethanol concentration at different timeframes in the presence of yeast using the variations in the setup of graphite-graphite electrochemical cells viz. first setup consists of the addition of ferricyanide in the graphite-graphite electrochemical cells at room temperature, second setup consists of the addition of 0.2 mg/mL of ferricyanide in the graphite-graphite electrochemical cells at 28^{o}C and the third setup consists of 2 minutes nitrogen purging at room temperature without ferricyanide treatment. All the experimental setups were supplemented with 6.5% of glucose in the PYN media and 4V of electricity was supplied. The absorbances of glucose using TBP method at 595 nm were recorded for each experimental setup at the time interval of 24 hrs, 48 hrs, 72 hrs, 96 hrs and 120 hrs and then the equation from linear regression analysis of the standard ethanol curve was used to calculate the respective values of the ethanol concentrations for each setup (n=3, $P<0.05$).

SN	Sample	Time (hrs)	Mean ethanol concentration (mg/mL) using the TBP method in reference to the standard (±SD)	Percentage of ethanol
	Graphite-Graphite with ferricyanide @ 0.2 mg/mL in 300 mL solution at room temperature			
1	Anode (Graphite)	24	13.71 ± 0.08	1.37
		48	13.80 ± 0.05	1.38
		72	14.79 ± 0.04	1.48
		96	16.68 ± 0.03	1.67
		120	17.64 ± 0.03	1.76
2	Cathode (Graphite)	24	10.95 ± 0.05	1.09
		48	11.02 ± 0.06	1.1
		72	11.39 ± 0.10	1.14
		96	11.73 ± 0.05	1.17
		120	12.32 ± 0.04	1.23
	Graphite-Graphite with ferricyanide @ 0.2 mg/mL in 300 mL solution at 28°C			

3	Anode (Graphite)	24	14.92 ± 0.05	1.49
		48	15.66 ± 0.08	1.57
		72	16.74 ± 0.13	1.67
		96	41.48 ± 0.10	4.15
		120	21.82 ± 0.12	2.18
4	Cathode (Graphite)	24	10.65 ± 0.07	1.06
		48	10.83 ± 0.06	1.08
		72	10.89 ± 0.04	1.09
		96	11.21 ± 0.04	1.12
		120	11.60 ± 0.04	1.16
	Graphite-Graphite with a nitrogen purge of 2 minutes			
5	Anode (Graphite)	24	17.92 ± 0.05	1.79
		48	19.46 ± 0.08	1.95
		72	24.98 ± 0.06	2.5
		96	48.23 ± 0.07	4.82
		120	27.67 ± 0.08	2.77

6	Cathode (Graphite)	24	16.68 ± 0.07	1.67
		48	17.33 ± 0.06	1.73
		72	17.43 ± 0.06	1.74
		96	17.98 ± 0.01	1.8
		120	18.79 ± 0.05	1.88

From **Table 5**, when culture was kept constantly at 28^{o}C which is the optimal temperature for the growth of yeast during the ferricyanide treatment, a significant increase in the metabolism of yeast was observed in the graphite anode at 96 hrs. At 96 hrs, the ethanol concentration increased to 41.48 ± 0.10 mg/mL from the concentration of 16.74 ± 0.13 mg/mL at 72 hrs. At 120 hrs the drop in ethanol concentration was seen which is 21.82 ± 0.12 mg/mL.

Similarly, from **Table 5** it was observed that without the ferricyanide treatment when a nitrogen purge was given at room temperature before the supply of electricity, then the optimal production of ethanol in the yeast was seen at 96 hrs in the anode. At 96 hrs, the ethanol concentration increased to 48.23 ± 0.07 mg/mL from the concentration of 24.98 ± 0.06 mg/mL at 72 hrs. At 120 hrs the drop in ethanol concentration was seen which is 27.67 ± 0.08 mg/mL. As a negative control, the effect of temperature on ethanol production at 28^{o}C was carried out further without electricity.

Table 6: Effect on the ethanol production by yeast without electricity, with shaking and using a bioreactor. The table represents data on ethanol production at different timeframes in the presence of yeast and the absence of electricity. Data from three different setups are shown viz. the first setup

was carried out without electricity and without shaking at 28°C, the second setup was carried out without electricity but a shaking of 100 rpm was given at 28°C and the third setup was carried out in a bioreactor giving temperature of 28°C pH of 6.5 and agitation of 100 rpm. All the experimental setups were supplemented with 6.5% of glucose in the PYN media. The absorbances of ethanol using TBP at 595 nm were recorded for each experimental setup at the time interval of 24 hrs, 48 hrs, 72 hrs, 96 hrs and 120 hrs and then the equation from linear regression analysis of the standard ethanol curve was used to calculate the respective values of the ethanol concentrations for each setup ($n=3$, $P<0.05$).

SN	Sample	Time (hrs)	Mean ethanol concentration (mg/mL) using TBP method as described by Seo et al., 2009 and Miah et al., 2017 in reference to the standard (±SD)	Percentage of ethanol
1	Without electricity and shaking at 28°C	24	5.82 ± 0.04	0.582
		48	10.35 ± 0.37	1.03
		72	37.43 ± 0.06	3.74
		96	50.61 ± 0.07	5.01
		120	34.36 ± 0.09	3.44
2	Without electricity	24	6.43 ± 0.43	0.643

	and shaking at 100 rpm and 28°C	48	11.05 ± 0.07	1.1
		72	44.30 ± 0.04	4.43
		96	51.27 ± 0.05	5.13
		120	33.74 ± 0.06	3.37
3	3 liters culture in a bioreactor at pH 6.5,100 rpm and 28°C	24	13.71 ± 0.10	1.37
		48	49.04 ± 0.03	4.9
		72	58.76 ± 0.10	5.88
		96	32.29 ± 0.58	3.23
		120	22.66 ± 0.57	2.27

The data from **Table 6** showed the highest production of ethanol in the yeast in the bioreactor at 72 hrs. In the bioreactor, at 72 hrs, the ethanol concentration increased to 58.76 ± 0.10 mg/mL from the concentration of 49.04 ± 0.03 mg/mL at 72 hrs and at 96 hrs and 120 hrs the ethanol concentration was 32.29 ± 0.58 mg/mL and 22.66 ± 0.57 mg/mL respectively.

The second setup without the current supply where the shaking was done at 100 rpm at 28°C showed the second highest production of ethanol in the yeast at 96 hrs. In this second setup, at 96 hrs, the ethanol concentration increased to 51.27 ± 0.05 mg/mL from the concentration of 44.30 ± 0.04 mg/mL at 72 hrs and at 120 hrs the ethanol concentration was 33.74 ± 0.06 mg/mL.

The first setup without the current and without shaking done at 100 rpm at 28^{o}C showed the lowest production of ethanol in the yeast at 96 hrs. In this first setup, at 96 hrs, the ethanol concentration increased to 50.61 ± 0.07 mg/mL from the concentration of 37.43 ± 0.06 mg/mL at 72 hrs and at 120 hrs the ethanol concentration was 34.36 ± 0.09 mg/mL. In all three setups, the ethanol concentration dropped at 120 hrs as compared to 96 hrs.

There were also variations in glucose metabolism and ethanol production using ferricyanide treatment (0.2 mg/mL) in the graphite-graphite electrochemical cells at room temperature and at 28^{o}C as seen in **Table 2** and **Table 5**. Although the growth and activity of yeast in electrochemical cells are reported to be supported by ferricyanide as a mediator (Gunawardena et al., 2008; Baronian, 2002), the results showed that optimal temperature for yeast growth at 28^{o}C (Liu and Shen, 2008) also plays a significant effect in the activities of yeast in the electrochemical cells using the graphite-graphite electrode. Also, from **Table 2** and **Table 5,** it can be seen that 2 minutes of the nitrogen purge did not show a significant effect on the activities of yeast as compared to those without treatment.

Table 3 and **Table 6** showed that although the glucose metabolism and ethanol production in the yeast was seen using other general microbial techniques to culture yeast, the supply of electricity in the electrochemical cell showed better production glucose metabolism and ethanol production in the yeast as compared to the general microbial techniques. A similar increase in the activities of yeast was observed by other researchers when the electricity was supplied (Mattar et al., 2015; Nakanishi et al., 1998).

The sudden decrease of ethanol concentration observed at 120 hrs in the case of electrochemical cells in **Table 4** and **Table 5** including the general microbial techniques as shown in **Table 6** shows that the yeast may have utilized the ethanol for energy production via oxidative respiration and

gluconeogenesis when glucose as the carbon source got depleted (De et al., 2012; Lutstorf and Megnet, 1968; Wills, 1976).

4.4. Analysis of the compounds present in the sample through HPLC

The samples from glucose metabolism and ethanol production were compared with the glucose and ethanol standard using HPLC, the graph of which were presented in **Figure 7**.

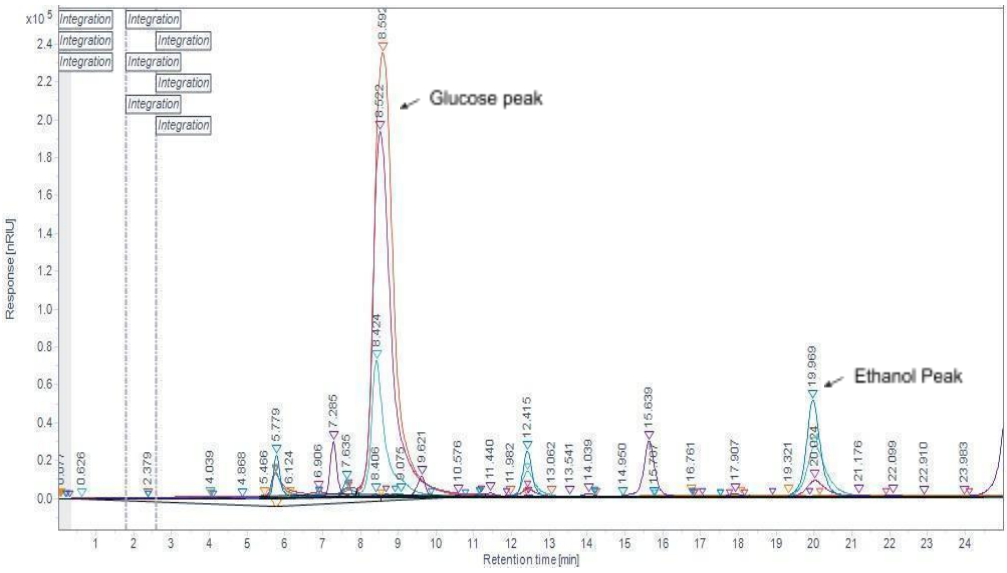

Figure 7: Combined peaks of glucose standard, ethanol standard and sample: The peaks represent combined refractive index signals from glucose standard, ethanol standard, and samples taken from the anode of the graphite-graphite electrode at 48 hrs and 96 hrs.

From **Figure 7** it was observed that a peak from the sample matched with the peak of the glucose and ethanol as standard and showed the presence of glucose and ethanol in the samples from the anode of graphite-graphite electrode. **Figure 7** as the qualitative measure to identify the presence of glucose and ethanol in the sample via HPLC gave a confirmatory result of the presence of ethanol and glucose in the sample (Picha, 1985).

4.5. Identification of PCR product

The PCR product obtained using the primers was observed using gel electrophoresis as shown in **Figure 8**.

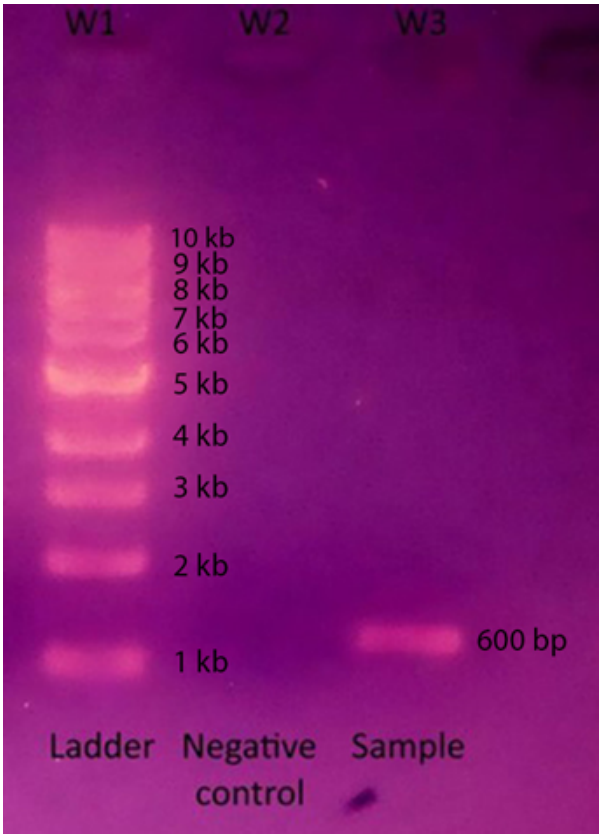

Figure 8: Identification of PCR product. The sample was run on 0.8% agarose gel having an EtBr concentration of 50µg/mL and 3µl of PCR product for 18s rRNA was loaded in the well along with negative control and 1kb ladder.

From **Figure 8** it was observed that the size of the 18s rRNA PCR product was at around 600 bp between the last two ladders each having a 1 kb fragment. The 600 bp amplicon obtained from 18s rRNA PCR in **Figure 8** was also reported by other researchers in the yeast but the role of this amplicon is yet to be determined (Banos et al., 2018).

4.6. Protein determination using SDS-PAGE

The protein determination using SDS-PAGE was recorded as per **Figure 9**.

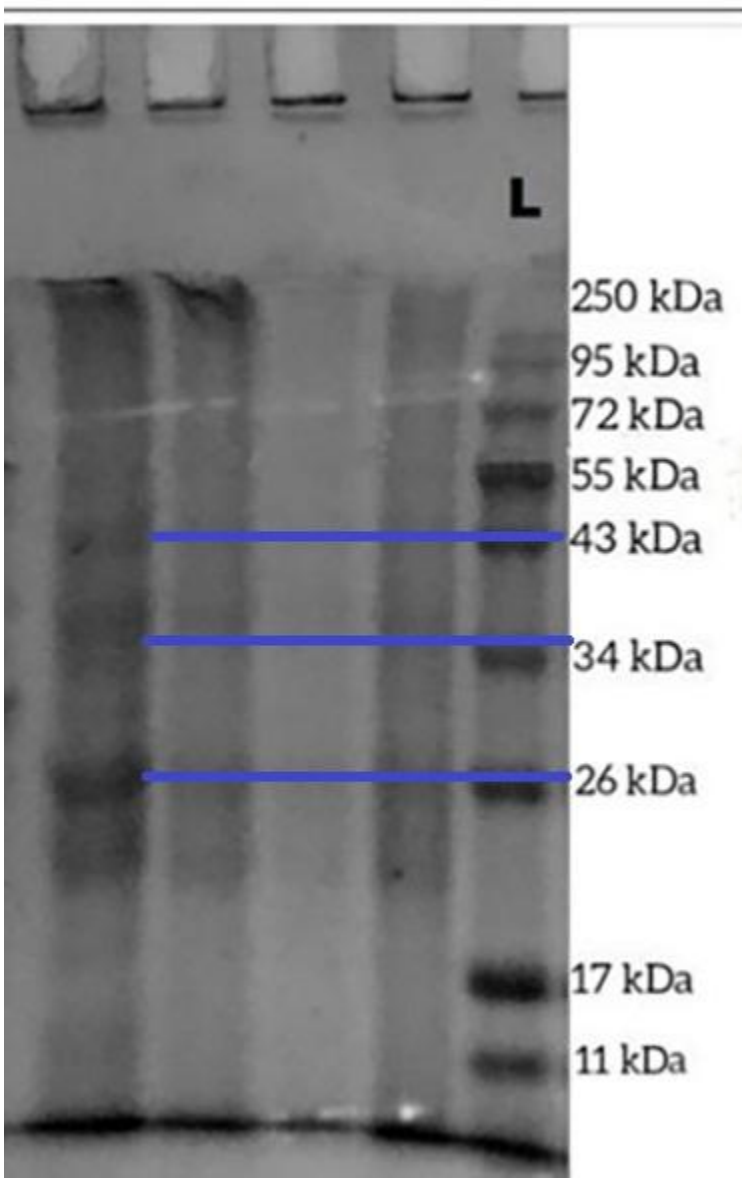

Figure 9: Protein determination using SDS-PAGE: The ladder of size range 11-250 kDa was used and represented by letter L whereas, from left hand side, the first lane represents sample from without electricity, the second lane represents sample from platinum as cathode and third lane represent graphite as anode.

From **Figure 9** it was observed that a protein of size of about 26kDa, 34kDa and 43 kDa was seen in the sample. The protein fragment of size about 26 kDa (Lue et al., 1995; Marzioch et al., 1994), 34 kDa (Santocanale et al., 1995; Leber et al., 1994) and 43 kDa (Braun et al., 2011; Leber et al 1994), and from yeast was also reported by several researchers but again its role and identity are yet to be determined.

CHAPTER 5. CONCLUSION

This research explores the effect of 4V DC current in glucose metabolism and ethanol production of yeast isolated from murcha of Nepal. The results are interesting in the way that they clearly show the effect of different electrochemical cells and setups on the overall glucose and ethanol metabolism. This kind of research helps to provide necessary data and insights into the research of microbial fuel cells. This research also compares the effect of electricity with other general microbial culture techniques of the yeast and clearly indicates that the supply of electricity can be an alternative to enhance the production of ethanol. These findings can be utilized by the alcoholic beverage industries as well as other research organizations to enhance the capacity of ethanol production. This research also helps to provide insights to solve a global challenge of organic waste decomposition and the production of useful byproducts by using yeast. The research also highlights the potential to screen and identify the salt and ethanol-tolerant yeast from the murcha which has been used by various societies of Nepal since the ancient age. These yeasts, if identified using genetic methods and if researched further can establish a new industry of yeast production in Nepal using the possible new strains from the murcha. As we know the challenge of waste utilization and energy production is growing day by day in this world and humans need to explore new ideas and potential sources to solve these challenges. Hence, these kinds of research will still be a help as a drop in the ocean to solve the problem. With time the availability of new techniques and exploration of new coated and uncoated electrochemical cells will grow and one-day findings from these kinds of research will definitely help to establish a new generation of industries that can efficiently produce by-products like ethanol and electricity from microbial fuel cells. Further analysis of 18s rRNA fragments and exploration of the role of 55 kDa protein in the impact on the efficiency of yeast in the future is recommended via this research. Also, it is recommended that further exploration into the combination of different electrochemical cells using different parameters is needed to get a concrete conclusion about the performance of yeast.

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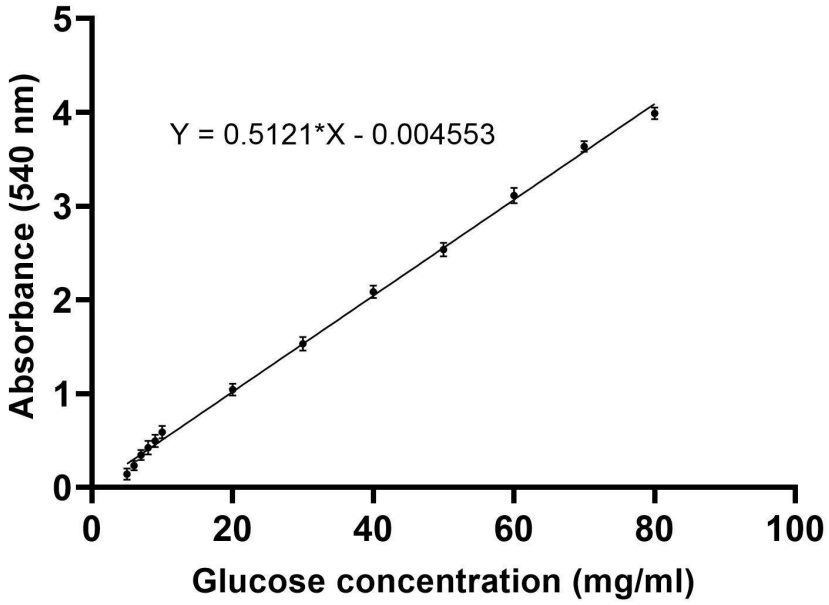
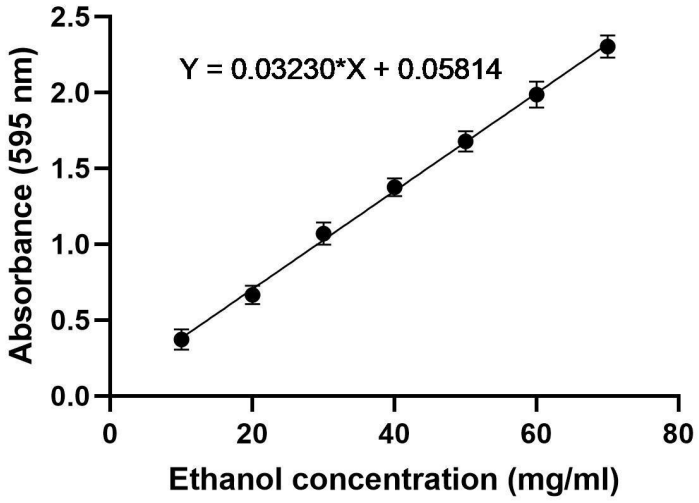
APPENDIX



Appendix I: Standard glucose curve. The glucose of different concentrations viz. 5 mg/mL, 6 mg/mL, 7 mg/mL, 8 mg/mL, 9 mg/mL, 10 mg/mL, 20 mg/mL, 30 mg/mL, 40 mg/mL, 50 mg/mL, 60 mg/mL, 70 mg/mL and 80 mg/mL were taken and using DNSA method absorbances were recorded using UV spectrophotometer at 540 nm. The graph of absorbance was plotted on the y-axis against the different concentrations of glucose on the x-axis for the linear

regression analysis which yielded an equation of Y=0.5121*X-0.004553. Triplicate data were taken to plot the graph using GraphPad Prism (n=3, $P<0.0001$).



Appendix II: Standard ethanol curve. The ethanol of 10 mg/mL, 20 mg/mL, 30 mg/mL, 40 mg/mL, 50 mg/mL, 60 mg/mL, and 70 mg/mL concentrations were taken, and using the TBP method absorbances were recorded using a UV spectrophotometer at 540 nm. The graph of absorbance was plotted on the y-axis against the different concentrations of ethanol on the x-axis for the linear regression analysis which yielded an equation of Y = 0.03230*X + 0.05814. Triplicate data were taken to plot the graph using GraphPad Prism (n=3, P<0.0001).

Appendix III: Composition of DNSA

Ingredients	Amount
NaOH (2N)	20 ml
Dinitrosalicylic acid	1g
Sodium potassium tartarate tetrahydrate	30 g

Preparation: Dissolve 1.0 gm of 3,5-dinitrosalicylic acid in 50 ml of reagent grade water. Add slowly 30.0 gms sodium potassium tartrate tetrahydrate. Add 20 ml of 2 N NaOH. Dilute final volume upto 100 ml with deionized water.

APPENDIX IV: SDS-PAGE GEL, REAGENT AND RUNNING PROTOCOL

1. 10% fresh Ammonium persulfate (APS)

Ingredients	Amount
APS	25mg
D/W	250ml

Preparation: Fresh APS was prepared each time by mixing the required amount of APS with

D/W in an Eppendorf tube.

2. Running (Electrode) buffer

Ingredients	Amount
Tris (39mM)	4.724gm
Glycine (8mM)	3.603gm
SDS (0.1%)	0.37gm
pH	8.4 ‡ 0.2
D/W	1000mL

Preparation: Above components were mixed in 800mL D/W with a magnetic stirrer and pH was adjusted to 8.4. Then, the volume was maintained to 1000 mL

3. Upper Tris (pH 6.8)

Ingredients	Amount
0.5 M Tris base	3.03gm
D/W	50 ml

Preparation: Tris base was dissolved in half the volume of water and pH was maintained at 6.8 with 1N HCI. The final volume was made up to 50 mL with D/W.

4. 10 % SDS

Ingredients	Amount
SDS	10gm
D/W	100 ml

Preparation: 10gm of SDS was completely dissolved in 100 ml D/W to make a 10% SDS

5. Loading (sample) buffer

Ingredients	Volume (mL)
Upper tris buffer pH 6.8	1.25
10% SDS	3
Glycerol	4.75
6-Mercaptoethanol	0.5
0.1% Bromophenol blue	0.5

6. Staining solution

Ingredients	Amount
Comassie brilliant blue-250	0.125gm
Glacial acetic acid	50 ml
Methanol	10 mL
D/W	40 ml

7. Destining solution I

Ingredients	Volume
Methanol	50 ml
Acetic acid	10 ml
D/W	40 ml

8. Destaining solution II

Ingredients	Volume
Methanol	5 ml
Acetic acid	7 ml
D/W	88 ml

Preparation: Following components were mixed properly in a conical flask and it was stored at room temperature.

9. Resolving gel (12%)

Ingredients	Volume
Acrylamide (40%)	2.4 ml
Resolving buffer	2 ml
D/W	3.5 ml
10% APS	80 uL
Tetramethylethylenediamine (TEMED)	5 ul

10. Stacking gel (4%)

Ingredients	Volume
Acrylamide (40%)	0.30ml
Resolving buffer	0.75 ml

D/W	1.92 ml
10% APS	30 ul
TEMED	2 ul